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PRINCIPAL INVESTIGATOR: Dr. Kounsouke Watabe

CONTRACTING ORGANIZATION: Southern Illionis University
Springfield, IL 62794

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INTRODUCTION

Adenocarcinoma of the prostate is currently the most prevalent cancer in men in the United States and represents 36% of all cancers among men (1). It is estimated that more than 210,000 new cases are diagnosed and 32,000 patients succumb to this disease every year (1). Although patients with localized lesions can be cured by radical prostatectomy or radiotherapy, more than 90% of cancer deaths are attributed to metastatic disease (2). Even those patients who have localized cancer and have been "successfully" treated with surgery often experience "recurrent" disease after many years. In the last two decades, numerous chemotherapeutic agents have been studied. However, the overall results have been quite disappointing. The failure of the current approach to develop an anti-cancer drug for prostate cancer suggests that we need essentially a new approach by defining a specific target molecule in this cancer (3). Traditional screening of anti-cancer drugs has been mostly dependent on growth inhibition assay for cancer cells. However, targeting a specific gene with well-defined clinical rationale will provide a better chance of developing a more effective therapeutic agent.

Fatty acid synthase, FAS, is expressed at low or undetectable levels in most normal human tissues, with the exception of lactating breast and cycling endometrium. In contrast, elevated expression of FAS and abnormally active endogenous fatty acid synthesis are characteristics of many human cancers, and the up-regulation of FAS was related in most cases to poor prognosis (3,4). Although the biological basis for this phenotype alteration in cancer cells is not clearly understood, it represents an experimental strategy for cancer therapy because inhibition of FAS is selectively cytotoxic for tumor cells and causes apoptosis. How the inhibition of FAS leads to cell death is an intriguing question. Considering that almost none of the conventional chemotherapeutic agents are effective for prostate cancer, we turned our attention to natural and herbal products that have been used for cancer treatment in different geographic areas. After screening over 100 different herbal plants for their inhibitory effect on the FAS expression, we found that *S. virgaurea* and Cacalia delphiniifolia have strong suppressor activities on the FAS gene. The cytotoxic activity of both plants appears to be mediated by inhibition of FAS, which eventually leads to apoptosis. The most intriguing question is how *S. virgaurea* suppresses the expression of FAS. We hypothesize that the active component of *S. virgaurea* suppresses tumor growth by inducing apoptosis through inhibition of FAS and that this inhibitory effect on FAS is mediated by blocking the upstream signal of FAS gene expression. In this project, we plan to accomplish two specific aims: (i) define the mechanism of cytotoxic activity of *Solidago virgaurea,* and (ii) to examine the effect of the active component of *Solidago virgaurea* on tumorigenesis in an animal model of prostate cancer

BODY

Task 1a: We will first purify the active component of *S. virgaurea* through a series of column chromatography.

Because our previous experimental results indicate that the FAS inhibition assay is a rational screening device for new anti-neoplastic compounds, we set an assay system for screening anti-FAS activity. After screening over 100 different herbal plants for their inhibitory effect on the FAS expression, we found that an extract of *S. virgaurea* has strong suppressor activities on the FAS gene. To examine the effects of S. virgaurea on tumor cells the powdered plant was extracted with H2O with or without heat treatment at 90C, ethanol, or chloroform. The samples were then tested for their cytotoxic activities on human prostate tumor PC3 cells. We found that the extract prepared with H2O or ethanol showed strong cytotoxic activities regardless of the temperature during the extraction, while the chloroform extract had little effect on PC3 cells (Fig. 1 A). When the extracts were prepared from

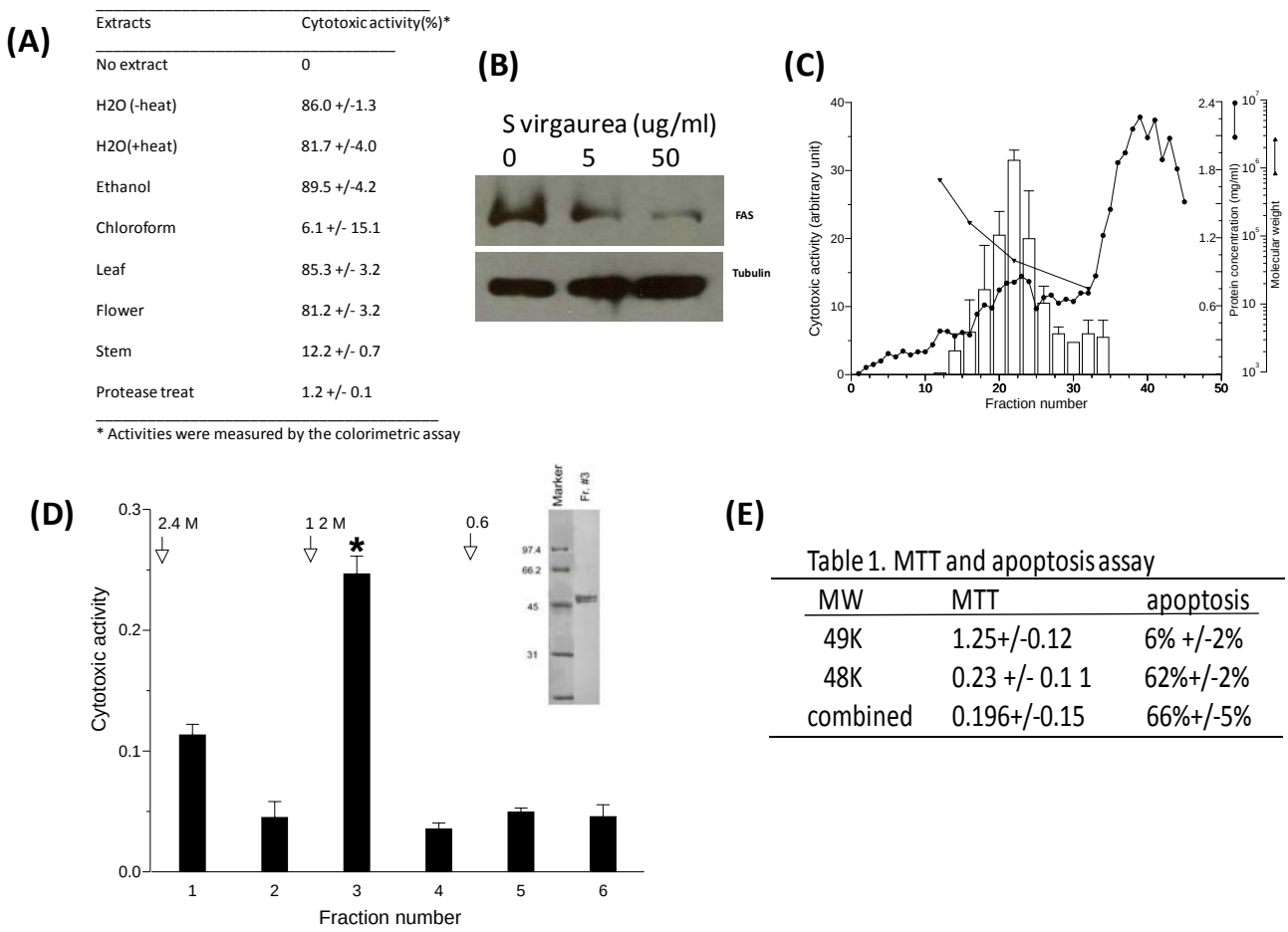
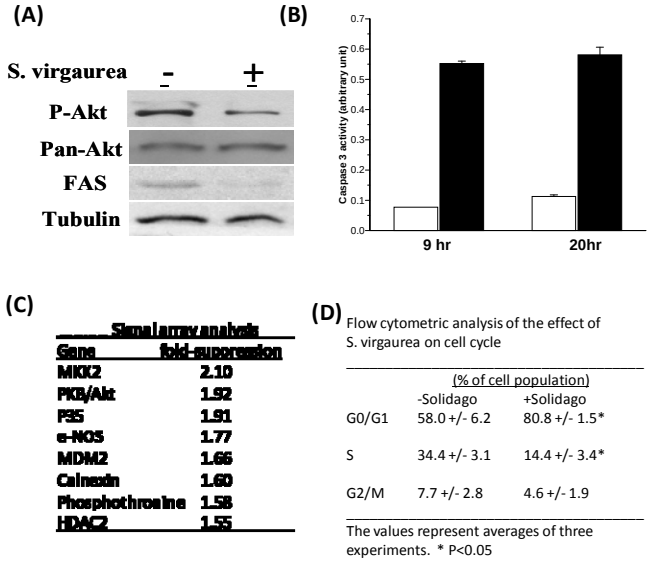


Fig. 1. Active component of S. virgaurea suppresses FAS expression and blocks cell cycle. (A) various parts of S. virgaurea were extracted with different medium and their cytotoxic activities were tested using PC3mm cells. (B) The extract of S. virgaurea was added in the culture of PC3mm and the expression of FAS was examined by Western blot analysis. (C) The extract was fractionated by G100 column and the cytotoxic activity of each fraction to tumor cells was examined. (D) The active fractions of G100 column chromatography were pooled, dialyzed and applied to an HIC column, which was washed and eluted with ammonium sulfate buffer with the indicated salt concentrations. The eluted fractions were assayed for their cytotoxic activities and subjected to SDS-polyacrylamide gel electrophoresis (inset). (E) The 48K protein was extracted from SDS gel, renatured and added into the culture of PC3mm cells followed for 48 hrs and they were subjected to MTT and apoptosis assays.

different parts of the plant, we found that the leaves and flowers had significantly higher activities than the stem. The extract of S. virgaurea with H2O indeed strongly suppressed the expression of FAS as indicated by the results of Western blot (Fig. 1B). We then attempted to further purify the cytotoxic activity of *S. virgaurea* using various chromatographic media and found that a combination of heat-treatment followed by column chromatography of G100 and methyl-HIC (BioRad) can effectively purify the activity. The crude extract of *S. virgaurea* was heated at 80°C for 5min followed by centrifugation. The supernatant was concentrated by the Amicon concentrator and applied on a G100 column (Fig. 1C). The active fraction of G100 was then applied onto the HIC column which was

sequentially eluted with 2.4, 1.8, 1.2, 0.8, 0.6 and 0.3M $(NH_4)_2SO_4$ (Fig. 1D). When each fraction was dialyzed and assayed, we found that the cytotoxic activity was eluted with 1.2 M $(NH_4)_2SO_4$. After repeating the purification steps with G100-sephadex and the HIC chromatography, the final HIC fraction was analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig.1D (insert), the active fraction eluted from the HIC column contained two species of proteins that had close molecular weights around 47-49 kD. We have also tried various traditional column chromatographies including DEAE, HA, phosphate, ConA and heparin agarose. However, these column systems did not retain the active component under all tested conditions. We also tried to purify the protein by using HPLC with C16 column. However, we were not able to recover any activity. We then attempted to purify the two proteins directly from SDS PAGE. The partially purified fraction was run on two lanes of a polyacrylamide gel. After electrophoresis, one lane was cut and proteins were visualized with silver staining. Using the stained lane as a guide, two bands in the unstained gel were excised and gels were extensively washed with buffer to remove SDS and re-nature the proteins. Proteins were then eluted by electrophoresis in a dialysis bag followed by concentration using Centricon P10 which has a molecular weight cut-off at about 10,000. Each concentrated protein and their combinations were then assayed for cytotoxicity. As shown in Fig. 1E, we found that the protein with a lower molecular weight (48 kD) had significantly more cytotoxic activity, suggesting that the active molecule is a single protein. Task 1a was accomplished.

Task 1b: We will examine the status of the FAS signaling pathway upon addition of *S. virgaurea*. We will also examine the expression of various signal molecules using the antibody microarray.



(C)

Signal array analysis	
Gene	fold-suppression
MKK2	2.10
PKB/Akt	1.92
P35	1.91
e-NOS	1.77
MDM2	1.66
Calnexin	1.60
Phosphothreonine	1.58
HDAC2	1.55

(D) Flow cytometric analysis of the effect of S. virgaurea on cell cycle

	(% of cell population)	
	-Solidago	+Solidago
G0/G1	58.0 +/- 6.2	80.8 +/- 1.5*
S	34.4 +/- 3.1	14.4 +/- 3.4*
G2/M	7.7 +/- 2.8	4.6 +/- 1.9

The values represent averages of three experiments. * P<0.05

Because the expression of FAS is known to be partly controlled by the Akt pathway, we have examined the effect of *S. virgaurea* on the phosphorylation status of Akt. Human prostate cell line, PC3mm, was cultured in the presence and absence of *S. virgaurea* for 24 hrs. The cells were harvested and the cell lysate was subjected to Western blot using pan- and phospho-specific antibodies (Fig. 2A). Our results indicate that *S. virgaurea* indeed strongly inhibited the phosphorylation of Akt as well as the expression of FAS. This inhibition was also accompanied by Caspase 3 activation as shown in Fig. 3. These results indicate that inhibition of FAS expression by S. virgaurea is partly due to the blockade of the

Fig. 2. Active component of S. virgaurea induces cell cycle arrest and apoptosis in prostate cancer cells. (A) PC3mm cells were treated with or without the active component of S. virgaurea for 24 hrs and the cell lysate was subjected to Western blot analysis using antibodies to phospho-Akt, pan-Akt, FAS and tubulin. (B) PC3 cells (5 x 10^6 cells) were mixed with (closed bar) or without (open bar) the active fraction of S. virgaurea in 1ml of RPMI medium for 9 and 20 hours at 37°C. The cells were then harvested and the cell lysates were tested for Caspase-3 activity by ApoAlert kit (Clontech). Values are mean +/- SD of triplicate measurements. (C) Active fraction was added into the PC3mm culture and they were incubated for 48 hrs. The cell lysates were prepared and they were subjected to Panorama antibody microarray analysis. (D) The active fraction was added to PC3mm culture, and after 48 hrs of incubation, they were suspended and examined for cell cycle by FACS.

Akt pathway and that this blocking induces Caspase 3-dependent apoptosis pathway.

We also performed an antibody array analysis for tumor cells with or without treatment of S. virgaurea using Panorama Ab microarray system (Sigma Aldrich Co) which contains antibodies to various signaling molecules. The cells were lysed and proteins were labeled with Cy3 or Cy5 followed by hybridization to the antibody array slides. Fig.2C summarizes the list of signaling molecules that are significantly suppressed in the tumor cells, PC3mm, that were treated with S. virgaurea. It should be noted that Akt was also identified as a suppressed protein by this analysis. We then tested the effect of S. virgaurea on cell cycle by FACS analysis. As shown in Fig. 2D, we found that the treatment of prostate cell with S. virgaurea indeed significantly suppressed the population of S phage while increased the population in G0/G1 phase. These results indicate that S.virgaurea inhibits FAS expression followed by blocking cell cycle and induces apoptosis by activating the caspase pathway. Task 1b is considered to be accomplished.

Task 1c. **We will examine the status of Malonyl-CoA, ceramide and expression of the pro-apoptotic genes, BNIP3, DAPK2 and TRAIL as well in response to *S. virgaurea*.**

We have previously shown that inhibition of FAS expression by shRNA accumulated ceramide and induced BNIP3, DAPK2 and TRAIL (Fig. 3A). We expected *S. virgaurea* to show a similar effect on prostate tumor. To test this possibility, we have tested the effect of S. virgaurea on the expression of BNIP3 and DAPK2 by qRT-PCR. As shown in Fig. 3B, we found that S. virgaurea indeed up-regulated the expression of both BNIP3 and DAPK2, suggesting that apoptosis induced by S. virgaurea is mediated by up-regulation of these pro-apoptotic genes. To further verify this apoptotic pathway by S. virgaurea, we examined the effect of purified 48KD protein on the expression of BNIP3. As shown in Fig. 3C and D, we found that 48KD protein indeed

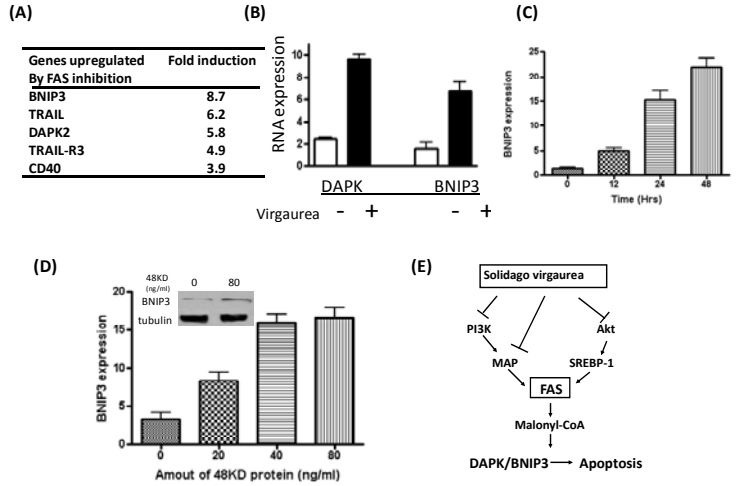


Fig. 3. 48KD protein up-regulates pro-apoptotic genes. (A) Cells were treated with or without siRNA-FAS for 48 hrs and the cell lysate was subjected to protein expression array for signaling pathway (Kinexus). (B) PC3mm cells were cultured in the presence and absence of S. virgaurea for 48 hrs. The cells were then harvested and RNA was prepared. The expressions of DAPK and BNIP3 were examined by qRT-PCR. (C, D) The effect of the purified 48KD protein on the expression of BNIP3 in PC3mm cells at various time point (C) and doses (D) were examined by qRT-PCR and Western blot. (E) Possible mechanism of action of S. virgaurea.

significantly up-regulated BNIP3 expression in a time and dose dependent manner, indicating that the purified 48KD protein is the active anti-tumorigenic component of S. virgaurea. These results indicate that S. virgaurea suppresses PI3K/MAPK pathway as well as AKT/SREBP signaling that together suppress the expression of the FAS gene followed by inducing apoptosis by activating DAPK/BNIP3 genes (Fig. 3E). Task 1c was accomplished.

Task 2. To examine the effect of the active component of *Solidago virgaurea* on tumorigenesis in a transgenic animal model of prostate cancer

We have purified 48KD protein as described above and examined the efficacy of this protein on tumorigenesis using a xenograft model. Human prostate cancer cell line PC3mm which was "labeled" with the luciferase gene was injected into the dorsal flank of nude mouse, followed by the intraperitoneal injection of 48KD protein (0.3ug/kg) every 3 days for a period of 3 weeks. As shown in Fig. 4, we found that the treatment of the mice with 48KD protein significantly suppressed the growth of tumor cells without showing noticeable toxicity to the animals. Importantly, mice did not show any hepatic injury or weight loss due to the treatment. Therefore, the 48KD protein from S. virgaurea indeed has effective anti-tumor activity without notable toxicity. We were also planning to use

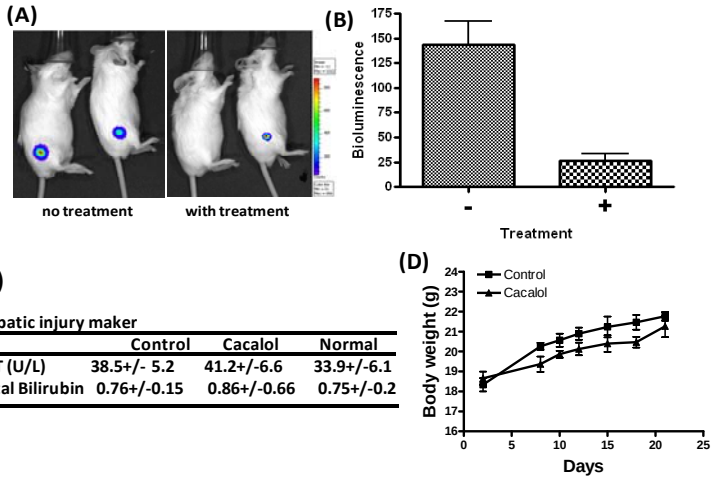


Fig. 4. Effect of 48KD protein on the tumor growth in mice. (A, B) PC3mm cells were injected into dorsal flank of SCID mice (n=5) followed by injection of 48KD protein via i.p (0.3ug/kg) every 3 days for a period of 3 weeks. The growth of tumor was monitored by Xenogen Bioimager. Note that treated tumor showed significant reduction of tumor volume. (A) representative image of mice. (B) luciferase activity of mice at Day 21. (C) At the end point, the blood from the mice was withdrawn and serum was tested for ALT and Bilirubin. (D) The body weight of mice was periodically measured and plotted.

TRAMP mouse to test the efficacy of the 48KD protein; however, due to difficulty in preparing a large quantity of this protein and also an unfortunate outbreak of mouse parvovirus, the progress in this task has not been accomplished. Although the fund will be provided by DOD for the next year, we will continue this project and also seek other funding to accomplish this goal.
Therefore, Task 2 is still in progress.

As we mentioned, we found that S. virgaurea had the anti-FAS activity when we were screening various traditional medicinal plants. During this screening phase, we also found that another plant,

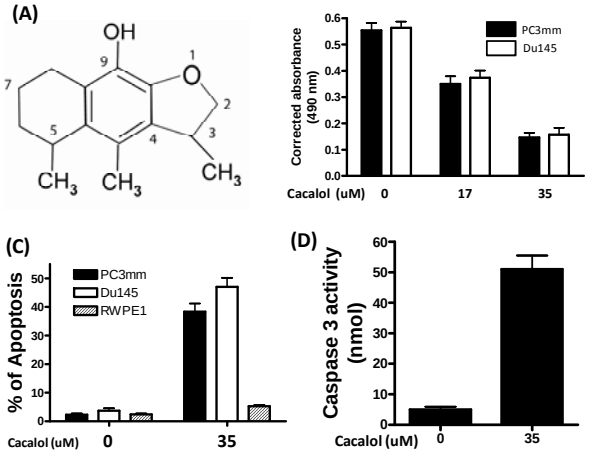
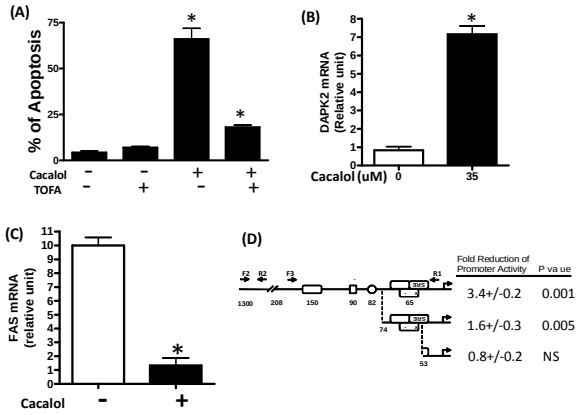


Fig. 5. Cacalol induces apoptosis in prostate cancer cells through activation of caspase pathway. (A) Chemical structure of cacalol. (B) Human prostate cell lines, PC3mm and Du145, were treated with or without various amounts of cacalol for 48 hrs at 37ºC. Cell survival was monitored by MTT assay. (C) PC3mm, Du145 and non-tumorigenic prostate line, RWPE1, were cultured and treated with or without cacalol at different concentrations (17 and 35 μM) for 48 hrs at 37ºC, and then apoptosis assay was performed by using *In situ* cell death TMR red kit. (D) PC3mm cells were treated with or without cacalol (35 μM) for 48hrs at 37ºC and then harvested. Cell lysates were tested for caspase 3 activity by ApoAlert Kit.

Cacalia deliphiniifolia, has strong anti-FAS activity. This plant is widely consumed as food in Asian countries, and it is also used as anti-inflammatory as well as anti-cancer drug. Although this was not originally included in our proposal, we thought pursuing this avenue is consistent with the overall goal of the project which is to identify natural compounds to block FAS activity for therapeutic utility. Therefore, we decided to study *Cacalia deliphiniifolia* in parallel. Because the extract of this plant showed strong anti-FAS activity, we purified the active component and we found that it was Cacalol (Fig. 5A). We then tested the cytotoxic effect of Cacalol on two human prostate cancer cell lines, PC3mm and DU145. As shown in Fig.5B, we found that Cacalol was indeed capable of significantly suppressing the growth of these cells and also induced apoptosis, while it did not affect the growth of non-tumorigenic prostate cells, RWPE1 (Fig. 5C). We also found that Cacalol can activate Caspase 3 activity (Fig. 5D).



In addition, to understand the mechanism of cacalol action, we tested the effect of TOFA which is the inhibitor of malonyl-CoA synthesis, a substrate of FAS. As shown in Fig. 6A, apoptosis-inducing effect of cacalol was significantly suppressed by TOFA, suggesting that inhibition of FAS by cacalol causes accumulation of malonyl-CoA , followed by inducing apoptosis. However, blocking the synthesis of malonyl-CoA overcomes the effect of cacalol. These results suggest that malonyl-coA plays a key role in this process of cacalol-inducing apoptosis. We also tested the effect of cacalol on the promoter activity of the FAS gene using a reporter plasmid as shown in Fig. 6D. The

Fig. 6. Cacalol inhibits FAS expression and induces apoptosis by activating DAPK2. (A) PC3mm cells were cultured and treated with or without cacalol (70 μM) in combination with TOFA (30 μM) for 48 hrs at 37ºC, and then the apoptosis assay was performed by using *In situ* cell death TMR red kit. (B) PC3mm cells were also treated with or without cacalol (35 μM) for 48 hrs at 37ºC. Cells were harvested and subjected to qRT-PCR using primers for the DAPK2 and β-actin genes. (C) PC3mm cells were treated with cacalol (35μM) for 48 hrs, followed by qRT-PCR analysis for the FAS gene expression. (D) For ChIP assay, MDA-MB231 cells were cultured with or without cacalol (35 μM) for 48 hrs. The cells were lysed and the lysate was pulled down with anti-SREBP-1 antibody. The DNA was then subjected to qPCR using non-specific (F2, R2) or SREBP binding site-specific primers (F3, R1).The ratio of the DNA was calculated based on cyclic threshold value for each reaction (right panel).

5'UTR of the FAS gene contains the three consensus binding sequence of SERBP1 which is a master regulator of the FAS gene. We found that deletion of these binding sites significantly reduced the effect of cacalol on the FAS promoter, suggesting that SREBP1 is involved in the process of inhibiting FAS expression.

Furthermore, we tested the effect of cacalol on the tumor growth in vivo. PC3mm cells were "labeled" with the luciferase gene followed by injecting them into SCID mice. Cacalol was given by gavage every other day for a period of 45 days. As shown in Fig. 7, we found that cacalol can significantly suppress the growth of prostate cancer cell, suggesting the utility of this compound for chemo-therapeutic and chemo-preventive application.

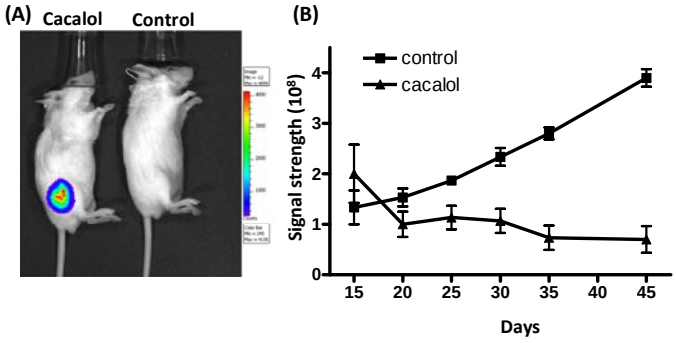


Fig. 7. Effect of cacalol on tumor growth in vivo. (A, B) PC3mm cells that were labeled with the luciferase gene, were injected subcutaneously into 4-weeks old male nude mice (n=5). Cacalol (30 mg/kg b.w.) was injected intraperitoneally to the mice every 4 days for 45 days. The tumor size was measured for each mouse twice a week for 8 weeks using bioluminescent imaging with an IVIS Imaging System (Xenogen).

KEY RESEARCH ACCOMPLISHMENTS

1. We have successfully purified the active component of S. virgaurea, which has 48kD of molecular weight with strong cytotoxic activity to prostate tumor cells. This protein was found to suppress growth and induce apoptosis in these human prostate cancer cells.
2. We have found that this active component blocked phosphorylation of Akt followed by inhibition of FAS expression. The results of our signal array data also indicate that MKK2 signaling is also activated.
3. This inhibition of Akt up-regulated the expression of pro-apoptotic genes, BNIP3 and DAPK, followed by activation of Caspase 3 and induction of apoptosis. We found that the purified 48kD protein significantly activated BNIP3 in time and dose dependent manners.
4. We found that the 48kD protein significantly suppressed the growth of tumor in our xenograft model in vivo, without showing notable toxicity.
5. We found another natural product, *Cacalia deliphiniifolia*, which blocks FAS expression and induces apoptosis. The active compound was purified and found to be identical with cacalol.
6. Cacalol induced apoptosis by inhibiting FAS and activating BNIP3/DAPK pathway followed by activation of caspase 3.
7. Cacalol significantly blocked the growth of prostate tumor cells in animals.

REPORTABLE OUTCOMES

Peer reviewed publications

(The following works were directly or partly supported by the current grant)

1. Wen Liu, Megumi Iiizumi, Hiroshi Okuda, Puspa Pandey and Kounosuke Watabe. S. virgaurea suppress the growth of prostate cancer cell growth by blocking the FAS gene. In preparation.
2. Wen Liu, Megumi Iiizumi-Gairani, Hiroshi Okuda, Aya Kobayashi, Misako Watabe, Sudha K. Pai, Puspa R. Pandey, Fei Xing, Koji Fukuda, Vishnu Modur, Shigeru Hirota, Kazuyuki Suzuki, Toshimi Chiba, Masaki Endo, Tamotsu Sugai, and Kounosuke Watabe (2011) KAI1 is engaged in NDRG1-mediated metastasis suppression in human prostate cancer. J. Biol. Chem. In press
3. Eiji Furuta, Hiroshi Okuda, Aya Kobayashi, Kounosuke Watabe (2010) Metabolic genes in cancer: Their roles in tumor progression and clinical implications . BBA Review on Cancer. 1805, 141-152
4. Furuta, E., Pai, SK., Zhan, R., Bandyopadhyay, S., Watabe, M., Iiizumi, M., Liu, W., Mo, Y-Y., Hirota, S., Hosobe, S.,Tsukada,T., Miura,K., Kamada, S., Saito, K. and Watabe, K. (2008) Fatty

acid synthase gene is up-regulated by hypoxia via activation of Akt and SREBP. *Cancer Res.* 68, 1003

5. Megumi Iiizumi, Sucharita Bandyopadhyay, Sudha K Pai, Misako Watabe, Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Ken Saito, Eiji Furuta, Wen Liu, and Kounosuke Watabe (2008). RhoC promotes metastasis via activation of Pyk2 pathway in prostate cancer. *Cancer Res.* 68(18):7613-20.
6. Megumi Iiizumi, Wen Liu, and Kounosuke Watabe. (2008) Drug development against metastasis-related gene and their pathways: A rationale for cancer therapy. *Biochim. Biophys. Acta. Cancer Review 1786*, 87-104.

Abstract/presentation

1. Eiji Furuta, Rui Zhan, Sucharita Bandyopadhyay, Shigeru Hirota, Sadahiro Hosobe, Misako Watabe, Sudha K. Pai, Megumi Iiizumi, Sonia Mohinta, Wen Liu, Kounosuke Watabe. Hypoxia induced ROS up-regulates the fatty acid synthase gene via Akt pathway in breast cancer cells. (2008) Annual meeting of American Association for Cancer Research. San Diego, CA
2. Wen Liu, Eiji Furuta, Misako Watabe, Kazutoshi Shindo, Megumi Iiizumi, Sudha Pai, Kounosuke Watabe. (2008) Inhibition of Fatty acid synthase and induction of apoptosis in human breast cancer cells by Cacalia deliphiniifolia Annual meeting of American Association for Cancer Research. San Diego, CA
3. Megumi Iiizumi, Sucharita Bandyopadhyay, Sudha K Pai, Misako Watabe,Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Ken Saito, Eiji Furuta, Wen Liu, and Kounosuke Watabe (2008) RhoC promotes metastasis but not growth of prostate tumor. Annual meeting of American Association for Cancer Research. San Diego, CA
4. Wen Liu, Sucharita Bandyopadhyay, Eiji Furuta and Kounosuke Watabe (2008) Role of tumor metastasis suppressor gene, NDRG1, in breast cancer progression. DOD Breast Cancer Research Program, Era of Hope 2008 Meeting. Baltimore MA
5. Fei Xing, Eiji Furuta, Misako Watabe, Sudha K Pai, Wen Liu, Puspa Pandey, Hiroshi Okuda, Aya Kobayashi, Megumi Iiizumi and Kounosuke Watabe Notch pathway is stimulated by hypoxia and promotes metastasis through activation of EMT and metalloproteinase. 2009 Annual meeting of American Association for Cancer Research. Denver Co.
6. Hiroshi Okuda, Eiji Furuta, Misako Watabe, Sudha K. Pai, Wen Liu, Aya Kobayashi, Fei Xing, Puspa Pandey, Megumi Iiizumi and Kounosuke Watabe The expression of metastasis suppressor gene, KAI1/CD82, is down-regulated by OCT4, SOX2 and NANOG in tumor stem cells of breast cancer. 2009 Annual meeting of American Association for Cancer Research. Denver Co.
7. Wen Liu[1], Eiji Furuta[1], Misako Watabe[1], Kazutoshi Shindo[2], Fei Xing[1], Sudha Pai[1], Hiroshi Okuda[1], Megumi Iiizumi[1], Puspa Pandey[1], Aya Kobayashi[1], Kounosuke Watabe[1]Inhibition of fatty acid synthase and induction of apoptosis in human breast cancer cells by Cacalia deliphiniifolia. 2009 Annual meeting of American Association for Cancer Research. Denver Co.
8. Eiji Furuta, Puspa R. Pandey, Hiroshi Okuda, Misako Watabe, Sudha K. Pai, Megumi Iiizumi, Wen Liu, Fei Xing, Aya Kobayashi, Kounosuke Watabe Resveratrol induces apoptosis by blocking enzymatic activity and destabilizing the protein of fatty acid synthase in breast tumor cells. 2009 Annual meeting of American Association for Cancer Research. Denver Co.
9. K. Watabe. Fatty acid synthase: a drugable metabolic oncogene for cancer therapy 2010. Department of Physiology, Southern Illinois University.
10. Aya Kobayashi, Hiroshi Okuda, Puspa R. Pandey, Misako Watabe, Sudha K. Pai, Shigeru Hirota, Fei Xing, Wen Liu, Bo Xia and Kounosuke Watabe BMP7 regulates dormancy and recurrence of prostate cancer stem cell in bone 2010 Joint Metastasis Research Society-AACR Conference on Tumor Microenvironment. Philadelphia PA

11. Wen Liu, Megumi Iiizumi, Puspa Pandey, Shigeru Hirota, Aya, Kobayahsi, Misako Watabe, Sudha Pai, Hiroshi Okuda, Fei Xing and Kounosuke Watabe. NDRG1 suppresses prostate tumor metastasis through modulation of the canonical Wnt pathway. 2011 IMPaCT conference. Orlando, Fl.
12. Aya Kobayashi, Hiroshi Okuda, Puspa Pandey, Misako Watabe, Sudha Pai, Fei Xing, Shigeru Hirota, Wen Liu and Kounosuke Watabe. Dormancy and recurrence of prostate cancer stem cell are regulated by bone morphogenetic protein 7 in bone. 2011 IMPaCT conference. Orlando, Fl.
13. Puspa R. Pandey, Hiroshi Okuda, Misako Watabe, Sudha K. Pai, Wen Liu, Aya Kobayashi, Fei Xing, Koji Fukuda , Shigeru Hirota, Tamotsu Sugai, Go Wakabayashi, Keisuke Koeda, Masahiro Kashiwaba, Kazuyuki Suzuki, Toshimi Chiba, Masaki Endo,Tomoaki Fujioka, Susumu Tanji, Yin-Yuan Mo, Deliang Cao, Andrew C. Wilber and Kounosuke Watabe. Up-regulation of lipogenic genes, SREBP-1, ACC, ACLYL and FAS, confers advantages of proliferation and survival to cancer stem-like cells in Ductal carcinoma in situ 2011 Annual meeting of American Association for Cancer Research. Orlando FL

Employment

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CONCLUSIONS

Solidago virgaurea has traditionally been used as an anti-inflammatory and anti-cancer medicine in several areas in the world. During the screening of anti-prostate drugs, we found that S. virgaurea has strong inhibitory activity to FAS expression. We have successfully purified the active component of S. virgaurea. The factor is 48kD protein with strong cytotoxic activity to prostate cancer cells. We also found that this protein can significantly suppress Akt and MKK2 signaling as well as FAS expression followed by inducing pro-apoptotic gene, BNIP3 and DAPK. More importantly, we found that the protein is indeed able to suppress tumor growth in mice, while the treatment did not show any apparent toxicity. These results strongly suggest that the 48kD protein is indeed the active component of anticancer activity of S. virgaurea. Therefore, we consider that the overall project is successfully completed and produced positive outcomes. While we were pursuing the project, we serendipitously found that another natural product, *Cacalia deliphiniifolia* also showed strong anti-FAS activity in prostate cancer cells. We identified the active compound as cacalol which has strong anti-oxidant activity. We also found that cacalol can suppress tumor growth in our animal model when it is given by oral administration, suggesting that this compound can be used for prevention of prostate cancer.

So what?

Our discovery of specific inhibition of FAS activity by the extract of *S. virgaurea* suggests a potential utility of this traditional medicine as a chemopreventive as well as therapeutic remedy for prostatic cancer. We have identified 48kD protein as an active component and, importantly, this protein was capable of significantly suppressing the tumor growth in mice, suggesting that this protein can be used as anti-cancer drug. There are four important implications in our findings. Firstly, we established a strong rationale to use S. virugaurea as chemo-preventive supplement for prostate cancer. Second, we anticipate that we can develop better anti-cancer peptides by further characterizing the 48kD protein.

Thirdly, further understanding of the mechanism of action of this protein may identify a novel therapeutic target for prostate cancer. Finally, our results suggest that cacalol can be used for chemo-prevention as a dietary supplement.

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KAI1 Gene Is Engaged in *NDRG1* Gene-mediated Metastasis Suppression through the ATF3-NFκB Complex in Human Prostate Cancer*[S]

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Wen Liu[‡], Megumi Iizumi-Gairani[‡], Hiroshi Okuda[‡], Aya Kobayashi[‡], Misako Watabe[‡], Sudha K. Pai[‡], Puspa R. Pandey[‡], Fei Xing[‡], Koji Fukuda[‡], Vishnu Modur[‡], Shigeru Hirota[§], Kazuyuki Suzuki[¶], Toshimi Chiba[¶], Masaki Endo[¶], Tamotsu Sugai[‖], and Kounosuke Watabe[‡1]

From the [‡]Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine, Springfield, Illinois 62794-9626 and the Departments of [§]Internal Medicine, [¶]Gastroenterology and Hepatology, and [‖]Diagnostic Pathology, Iwate Medical School, Japan

AQ:A,B

***NDRG1* and *KAI1* belong to metastasis suppressor genes, which impede the dissemination of tumor cells from primary tumors to distant organs. Previously, we identified the metastasis promoting transcription factor, ATF3, as a downstream target of NDRG1. Further analysis revealed that the *KAI1* promoter contained a consensus binding motif of ATF3, suggesting a possibility that NDRG1 suppresses metastasis through inhibition of ATF3 expression followed by activation of the *KAI1* gene. In this report, we found that ectopic expression of NDRG1 was able to augment endogenous *KAI1* gene expression in prostate cancer cell lines, whereas silencing NDRG1 was accompanied with significant decrease in *KAI1* expression *in vitro* and *in vivo*. In addition, our results of ChIP analysis indicate that ATF3 indeed bound to the promoter of the *KAI1* gene. Importantly, our promoter-based analysis revealed that ATF3 modulated *KAI1* transcription through cooperation with other endogenous transcription factor as co-activator (ATF3-JunB) or co-repressor (ATF3-NFκB). Moreover, loss of KAI1 expression significantly abrogated NDRG1-mediated metastatic suppression *in vitro* as well as in a spontaneous metastasis animal model, indicating that KA11 is a functional downstream target of the NDRG1 pathway. Our result of immunohistochemical analysis showed that loss of NDRG1 and KAI1 occurs in parallel as prostate cancer progresses. We also found that a combined expression status of these two genes serves as a strong independent prognostic marker to predict metastasis-free survival of prostate cancer patients. Taken together, our result revealed a novel regulatory network of two metastasis suppressor genes, *NDRG1* and *KAI1*, which together concerted metastasis-suppressive activities through an intrinsic transcriptional cascade.**

AQ: C

Although significant advances have been made in reducing mortality rates, the majority of cancer patients are still diagnosed at an advanced stage and ultimately die from a sequel of metastatic disease. Metastasis involves a complex process through which malignant cancer cells leave a primary organ site, journey to a distant site via circulation, and finally establish a clinically detectable mass in a distant organ, and therefore, the metastatic progression requires dysregulation of a series of genes and related signaling. Metastasis suppressor genes are negative regulators of metastasis, which inhibit metastasis but do not affect the ability of the transformed cells to generate a tumor at the primary site (1–4). More than 20 metastasis suppressors have been discovered so far, and they appear to be involved in several pivotal steps of metastasis, including invasion (NM23, DLC1, KAI1, and NDRG1), dissemination (KAI1, CD44), survival (BRMS1, caspase-8), and growth in distant sites (NM23, KAI1, RHOGD12, KISS1, RKIP, and MKK4/6) (4–7). However, the detailed molecular mechanism of how these genes are regulated and their functions are less elucidated.

AQ: D

AQ: E

KAI1, also known as CD82, was discovered initially from T-cell activation study and was identified later as a prostate-specific tumor metastasis suppressor gene (8–11). It is ubiquitously expressed in normal tissues with high mRNA levels in spleen, placenta, kidney, and prostate, whereas decrease or loss of its expression is constantly found in the clinically advanced cancers (12). Consistently, inverse correlations between KAI1 expression and the invasive and metastatic potential as well as poor survival of patients have been observed frequently in a wide range of malignancies (12). KAI1 belongs to the tetraspanin transmembrane protein superfamily, and it is found to be associated with other tetraspanins (CD9 and CD81), integrins (β1 and β2), immunoreceptors (MHCI and II, EWI1/PGRL, CD4, CD8, CD19, and CD46), growth factors and receptors (EGF and EGFR), as well as intracellular signaling proteins (PKC) (12). KAI1 was found previously to inhibit cell motility by regulating the biological activities of its associated proteins and/or reorganizing plasma membrane microdomains (12). This process occasionally induces apoptosis by releasing intracellular glutathione and accumulating intracellular reactive oxygen intermediates (13, 14). Moreover, we have demonstrated recently that KAI1 exerts its metastasis suppressor function by directly binding to DARC on endothelial cells, thereby inducing senescence signaling in tumor cells in circulation (15). Because mutations and loss of heterozygosity of the *KAI1* gene is rare, the down-regulation of this gene is not likely

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AQ: UU * This work was supported, in whole or in part, by National Institutes of Health Grants R01CA124650 and R01CA129000 (to K. W.). This work was also supported by U. S. Department of Defense Grants BC044370 and PC061256 (to K. W.).

ZSI [S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Methods" and a figure.

[1] To whom correspondence should be addressed. Tel.: 217-545-3969; Fax: 217-545-3227; E-mail: kwatabe@siumed.edu.

because of genetic alterations but is rather due to modulation of transcriptional and post-transcriptional regulation (16–20, 43). However, how KAI1 is down-regulated in metastatic cancer cells is largely unknown. Previously, we showed that p53 is able to bind to the *KAI1* promoter and turn on its transcription (21, 22). Other molecules involved in *KAI1* transcriptional regulation include NFκB, β-catenin/Reptin, Tip60/Fe65, N-CoR/TAB2/HDAC3 and AP-1 (23–28). Interestingly, these transcription factors were frequently found to coordinately regulate the expression of *KAI1* as either co-activator or co-repressor. Therefore, studies on these transcription factors may aid in elucidating the mechanism leading to KAI1 suppression and following metastatic progression.

NDRG1 (N-Myc downstream regulated gene 1) was originally isolated as a novel gene that was induced strongly during differentiation of colon carcinoma cell lines (29). Recent studies demonstrated that the *NDRG1* gene is controlled by a variety of factors and stimuli related to cancer progression, including oncogenes, tumor suppressors, hypoxic microenvironment, and hormone dysregulation (30, 31). Clinical studies also provided compelling evidence that reduced expression of the *NDRG1* gene was significantly associated with poor overall survival rate in pancreatic ductal adenocarcinoma, glioma, prostate, breast, and colorectal cancers (32). The significant inverse correlation of NDRG1 expression with the extent of metastasis in a clinical setting raised an important question as to whether the down-regulation of NDRG1 is cause or result of metastases. To address this issue, we overexpressed the *NDRG1* gene in a highly metastatic prostate cell line AT6.1 and implanted it into SCID mice (33). Our results indicate that NDRG1 has the ability to suppress the metastatic process of prostate cancer cells without affecting tumorigenicity *in vivo*. Similar metastasis suppressor effect of NDRG1 was also observed in other animal models including colon, bladder, and pancreatic carcinoma cells (34–36). Furthermore, NDRG1 was shown to suppress the invasive and angiogenic abilities of aggressive cancer cells (36–38). A recent study employing whole genome gene array analysis in examining the functions of NDRG1 in a number of different cancer cells strongly indicate the pleiotropic nature of NDRG1 in suppressing metastasis (39).

We previously performed the Affymetrix gene array analysis and found that NDRG1 suppressed metastasis of prostate tumor cells by inhibiting the transcription factor ATF3 (15). Consistent with our observation, Ishiguro *et al.* (40) showed that ectopic expression of ATF3 converted the low metastatic potential melanoma cell line to become highly metastatic. Moreover, ATF3 expression appears to be required for the maintenance of a high metastatic state of melanoma and colon cancer cells (41). ATF3 is a member of cAMP-responsive element binding protein (ATF/CREB) family of basic leucine zipper transcription factors (42). Emerging evidence suggests that ATF3 plays a critical role in metastatic progression in a cell context-dependent manner. To gain further mechanistic insight into the functional role of NDRG1, we sought to identify and characterize the possible downstream targets of ATF3 that are involved in tumor metastasis. The result of our bioinformatic analysis for the promoters of metastasis-related genes revealed that there were a number of genes whose promoter contained the ATF3 response consensus sequence, TGACGTCA. Among these, we identified proapoptotic gene *GADD153*/*CHOP10*, cell adhesion molecular E-selectin, tumor suppressor p53, and metastasis suppressor *KAI1* as potential targets of ATF3. These clues prompted us to examine a possible link between two metastasis suppressor genes, *NDRG1* and *KAI1*, through ATF3 in the current study.

EXPERIMENTAL PROCEDURES

Cell Culture—Human prostate cancer cell line PC3mm and rat prostatic carcinoma cell line AT6.1 were kindly provided by Drs. I. J. Fidler (The University of Texas MD Anderson Cancer Center, Houston, TX) and C. Rinker-Schaeffer (University of Chicago), respectively. The PC3mm/Tet[2] cell line was established previously as a derivative of PC3mm and contains a tetracycline-inducible suppressor. The human prostate cancer cell line DU145, prostate epithelial cell line RWPE1, and colon cancer cell line HT38 were obtained from the American Type Culture Collection (Manassas, VA). NDRG1- and KAI1-overexpressing stable clones of AT6.1 cells were established as described previously (33). For AT6.1/NDRG1/shKAI1, shRNA for KAI1 (Open Biosystems) or the vector alone was transfected into AT6.1/NDRG1 cells, and the puromycin-resistant clones were selected. The expression of KAI1 in the cloned cells was tested by RT-PCR. All cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), penicillin (100 units/ml), and 250 nM dexamethasone (Sigma) and grown at 37 °C in a 5% CO_2 atmosphere. For all transfection experiments, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's protocol.

Reporter Assay—The luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega, Madison, MI) and a luminometer (Berthold Detection Systems, Huntsville, Alabama). For each transfection experiment, the *Renilla* expression plasmid phRG-TK (Promega) was co-transfected as an internal control, and the promoter activities were normalized accordingly.

Immunohistochemistry—Immunohistochemical analysis on paraffin-embedded, surgically resected specimens of prostate and breast was carried out using anti-NDRG1 rabbit polyclonal antibody (from Dr. Commes) and anti-KAI1 antibody (kindly provided by Dr. Yoshie). Briefly, sections were deparaffinized, rehydrated, and heated at 80 °C for 20 min in 25 mM sodium citrate buffer (pH 9) for antigen exposure. They were then treated with 3% H_2O_2 to block endogenous peroxidase activity and further incubated with primary antibody for 1 h at 24 °C. After washing with Tris-buffered saline/0.1% Tween 20, the sections were incubated with horseradish peroxidase-conjugated rabbit-specific IgG (Dako). The sections were washed extensively, and DAB substrate chromogen solution was applied followed by counterstaining with hematoxylin.

Bioinformatics and Statistical Analysis—The GEO database (accession no. GSE21034, $n = 218$) was used to evaluate the clinical relevance of NDRG1 and KAI1 in prostate cancer. The data were log2-transformed, with the median set as zero and with S.D. set as one. Each patient was assigned to have positive

[2] The abbreviation used is: Tet, tetracycline.

or negative expression of each gene and was matched with metastasis-free survival. The gene expressions in normal, primary tumor, or metastatic sites in patients who have either metastatic or nonmetastatic disease were compared using Box-and-whisker plot analysis and evaluated by the Mann-Whitney test. The association between genes and clinical outcomes was calculated by the Pearson χ^2 test. The Kaplan-Meier method was used to calculate the overall survival rate, and prognostic significance was evaluated by the log-rank test. Multivariate analysis for the prognostic value of gene signatures was performed by the Cox proportional hazard regression model. For all statistical analysis, Prism and SPSS software were used. For *in vitro* experiments and animal studies, results are reported as mean ± S.D. (or mean ± S.E.) as indicated in the figure legends. Statistical significance was determined by a two-sided Student's test.

Animal Studies—4–6-Week-old SCID mice (Harlan Sprague-Dawley, Indianapolis, IN) were used for the spontaneous metastasis studies. 0.5×10^6 cells in 0.1 ml of PBS were injected subcutaneously into the dorsal flank of mice. Mice were monitored daily, and the tumor volume was measured as an index of the growth rate and calculated as (width + length)/$2 \times$ width $\times$ length $\times$ 0.5236. Mice were sacrificed 30 days after the inoculation of cells, and metastatic lesions were counted macroscopically.

RESULTS

NDRG1 Up-regulates Expression of KAI1 Gene in Prostate Cancer Cells—We first examined the effect of NDRG1 on the expression of the *KAI1* gene in two human prostate cancer cell lines, DU145 and PC3mm/Tet-FLAG-NDRG1 (referred to as PC3mm/Tet), by quantitative RT-PCR and Western blot. NDRG1 expression was induced by tetracycline treatment in PC3mm-Tet cells. Alternatively, the NDRG1 expression plasmid was transfected into DU145 cells. We found that the expression of *KAI1* gene was significantly up-regulated by ectopic expression of NDRG1 (Fig. 1*A*). To further examine whether NDRG1 has an effect on the transcription of the *KAI1* gene, we transfected the *KAI1* reporter plasmid into PC3mm/Tet and DU145 cells, which were then forced to overexpress the *NDRG1* gene. As shown in Fig. 1*B*, our results showed that NDRG1 expression indeed significantly augmented *KAI1* gene transcription. Moreover, when PC3mm cells were treated with Dp44mT, an NDRG1-specific agonist, the expression of the *KAI1* gene was also significantly elevated in a dose-dependent manner (Fig. 1*C*, *lower panel*). The increased KAI1 expression in the Dp44mT-treated cells was also confirmed by immunofluorescence staining (Fig. 1*C*, *upper panel*). Furthermore, we introduced NDRG1 shRNA into nonmalignant prostate epithelial cell RWPE1 or NDRG1 siRNA into colon carcinoma cell HT38 because both cell lines are known to express the *KAI1* gene. We found that the specific knockdown of *NDRG1* indeed significantly down-regulated the expression of the *KAI1* gene in these cell lines (Fig. 1*D*). We also examined the expression of *KAI1* and *NDRG1* in a panel of prostate and breast cancer cell lines ($n = 11$ and 39 for prostate and breast cancer cell lines, respectively). We found that *KAI1* mRNA expression was significantly correlated with *NDRG1* expression in multiple cancer cell lines ($p = 0.0014$ and 0.0004 for prostate (*upper panel*) and breast (*lower panel*) cancer cell lines, respectively, Fig. 1*E*), indicating that the expression of *NDRG1* and *KAI1* generally has a positive correlation in human prostate and breast cancer cells. To further examine the relevance of NDRG1 and KAI1 *in vivo*, we compared the expression of *Ndrg1* and *Kai1* in NDRG1 knock-out ($Ndrg1^{-/-}$) and syngenic wild type mice ($Ndrg1^{+/+}$). Consistent with the results of our *in vitro* studies, we found that *Kai1* mRNA expressed at a significantly lower level in *Ndrg1* knock-out mice compared with that in wild type mice (Fig. 1*F*).

NDRG1 Suppresses ATF3 Signaling-mediated KAI1 Inhibition in Prostate Cancer Cells—To identify the NDRG1 response sequence on the *KAI1* promoter, we generated a series of luciferase reporter plasmids containing up to −2900, −1118, −730, and −320 bases of the *KAI1* promoter, and luciferase reporter activities were measured in PC3mm/Tet cells with or without induction of NDRG1 expression. As shown in Fig. 2*A*, the deletion between −1118 and −780 bases of the *KAI1* promoter region almost completely abolished the ability of this promoter to respond to NDRG1. We also examined the *KAI1* promoter activity in DU145 cells, which was transfected with the NDRG1 expression plasmid and observed a similar result. These results suggest that the region between −118 and −780 contains an NDRG1 response sequence. When we examined the sequence of this region, we found three potential binding sites for ATF3, and one of these was a complete match to the consensus sequence. To further determine whether ATF3 binds to the *KAI1* promoter, ChIP assay was performed, and the results clearly showed that ATF3 indeed bound to the predicted region of the *KAI1* promoter, whereas induction of NDRG1 expression significantly blocked this binding (Fig. 2*A*, *lower panel*). Consistently, we found that silencing the expression of ATF3 by siRNA in PC3mm cells resulted in significant up-regulation of KAI1 mRNA expression (Fig. 2*B*). Therefore, our results strongly suggest that NDRG1 regulates KAI1 expression by modulating ATF3 signaling pathway. Surprisingly, however, we found that ectopic expression of ATF3 in PC3mm cells significantly up-regulated *KAI1* promoter activity and that the mutation of the ATF3-binding consensus sequence on the *KAI1* promoter abolished this responsiveness to ATF3 (Fig. 2*C*, *left panel*).

ATF3 is known to be a bidirectional transcription factor, which either activates or represses transcription dependent on its dimerized partner in a cell context-dependent manner (42). Interestingly, the NFκB subunit p50 was reported previously to be involved in a protein complex that was capable of inhibiting *KAI1* transcription (25), and p50 is also known to be able to form a dimeric complex with ATF family members (53). Therefore, we examined a possibility that ATF3 coordinates with p50 to repress *KAI1* transcription. We found that ATF3 was indeed pulled down with p50 (Fig. 2*C*, *right panel*) and works together to suppress *KAI1* promoter activity after co-transfection of ATF3 and p50 expression plasmids in PC3mm cells (Fig. 2*C*, *left panel*). However, ATF3 and p50 failed to down-regulate *KAI1* promoter activity when ATF3 binding sites were mutated (Fig. 2*C*, *left panel*), suggesting that p50 cooperates with ATF3 to suppress *KAI1* expression. To further verify this result, we

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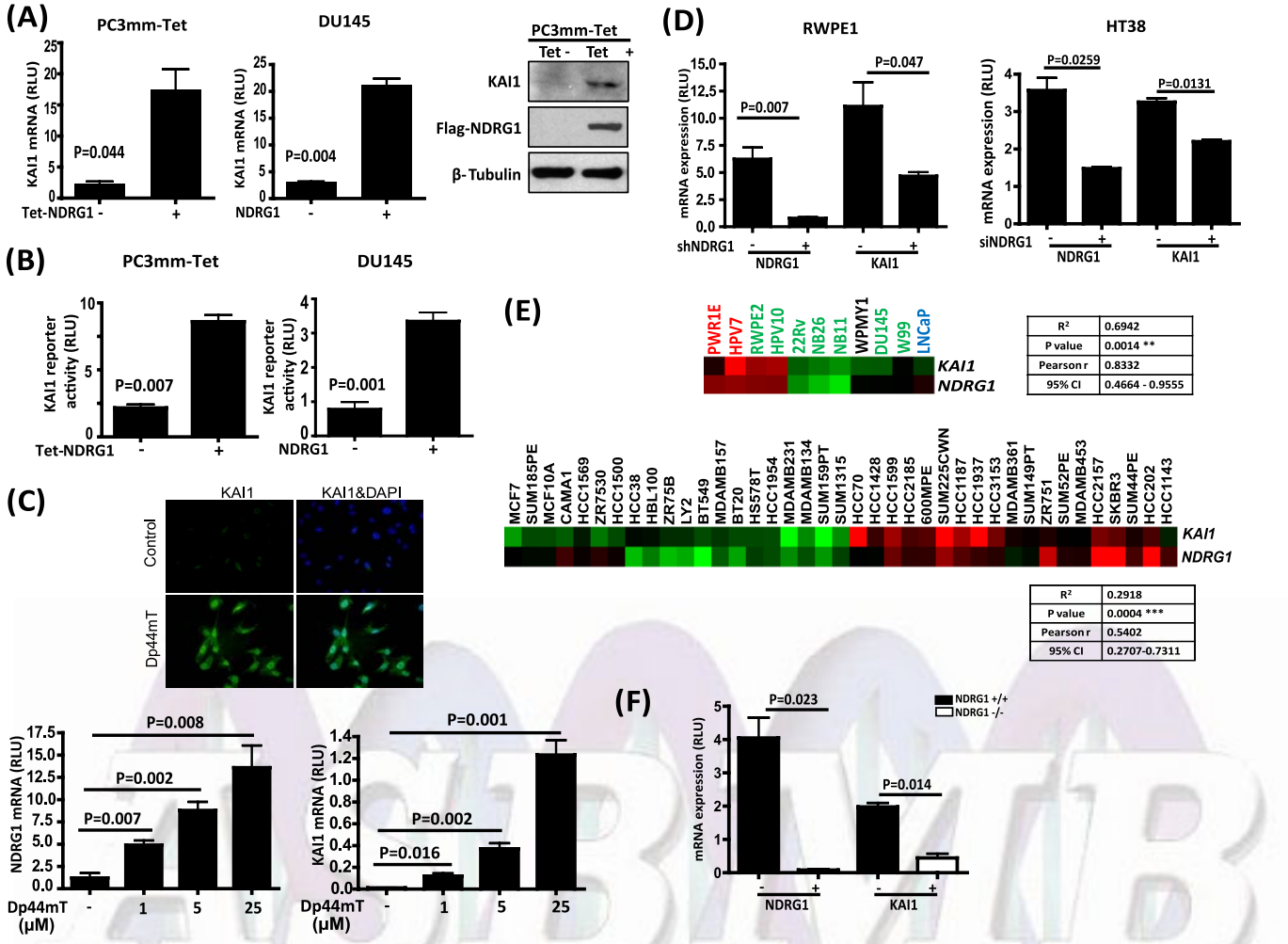

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AQ: OO FIGURE 1. **NDRG1 up-regulates KAI1 in prostate cancer cells.** *A* and *B*, PC3mm/Tet-FLAG-NDRG1 cells were treated with or without tetracycline. DU145 cells were transfected with the NDRG1 expression plasmid or an empty vector. The expression of *KAI1* mRNA was then examined by quantitative RT-PCR using primers for *KAI1* (*A*, *left* and *middle panels*). Western blot analysis was also performed to confirm *KAI1* expression (*A*, *right panel*). *B*, *KAI1* promoter-driven luciferase reporter plasmid was transfected into PC3mm/Tet-FLAG-NDRG1 and DU145 cells. Luciferase activity was assayed and normalized by internal *Renilla* luciferase activity. Values are means ± S.D. of triplicate measurements. *p* values are based on a two-sided Student's test. *C*, PC3mm/Tet-FLAG-NDRG1 cells were treated with different doses of Dp44mT. Expressions of *NDRG1* and *KAI1* mRNA were examined by quantitative RT-PCR. Values are means ± S.D. of triplicate measurements. *p* values are based on a two-sided Student's test. Immunocytochemical staining with antibody for KAI1 was also performed. *D*, RWPE1 cells were transfected with shRNA for NDRG1(*left panel*). HT38 cells were transfected with siRNA for NDRG1 (*right panel*). The expressions of NDRG1 and *KAI1* mRNA
AQ:PP, QQ were then examined by quantitative RT-PCR. *E*, *KAI1* expression correlates with *NDRG1* in a panel of prostate (*upper panel*, GSE9633) and breast (*lower panel*) cancer cell lines. The heat map depicts the relative expression of normalized values of *KAI1* and *NDRG1* for each cell line. *Green* indicates low expression, and *red* indicates high expression. The correlation of two genes was analyzed by linear regression. *F*, the expression of *Ndrg1* and *Kai1* mRNA in NDRG1 knock-out and
AQ: RR wild type mice were examined by quantitative RT-PCR. Values are means ± S.D. of triplicate measurements. *p* values are based on a two-sided Student's test.

AQ: S examined an effect of the p50 inhibitor, PDTC, on the expression of the *KAI1* gene in the presence or absence of NDRG1. As shown in Fig. 2*D*, either induction of NDRG1 or inhibition of p50 significantly up-regulated *KAI1* transcriptional activity in the PC3mm cell, and a combination of NDRG1 and PDTC further up-regulated *KAI1* promoter activity. However, neither of the treatments induced suppression of *KAI1* promoter activity when the ATF3 consensus binding site was mutated. These results strongly support our notion that NDRG1 up-regulates KAI1 by blocking the suppressor activity of the ATF3-p50 complex on the *KAI1* promoter. Notably, it was reported previously that p50 dimerized and bound to a region between −6631 and −6996 bp upstream of the *KAI1* gene transcription start site (23, 24). To further corroborate that p50 is involved in ATF3-mediated *KAI1* transcriptional regulation, we performed a ChIP assay by precipitating the p50-chromatin complex followed by quantitative PCR analysis with the pairs of primers specific to the region of ATF3 binding site. We found that p50 indeed bound to the proximal region of ATF3 binding site on the *KAI1* promoter by forming a complex with ATF3, which was negatively regulated by NDRG1 (Fig. 2*E*).

ATF3 can form a complex with various other members in the ATF family such as ATF2 and ATF4, as well as AP-1 proteins (27, 28). Interestingly, we found that ATF3, in combination with the AP-1 family protein JunB, significantly enhanced KAI1 transcriptional activity compared with each alone (Fig. 2*F*). Taken together, our result indicates that ATF3 modulates KAI1 expression through an intrinsic mechanism that is dependent on the dynamic cellular context of tumor progression.

KAI1 Is Involved in Downstream Signal of NDRG1-mediated Metastasis Suppression—The positive regulatory role of NDRG1 in KAI1 expression prompted us to examine the func-

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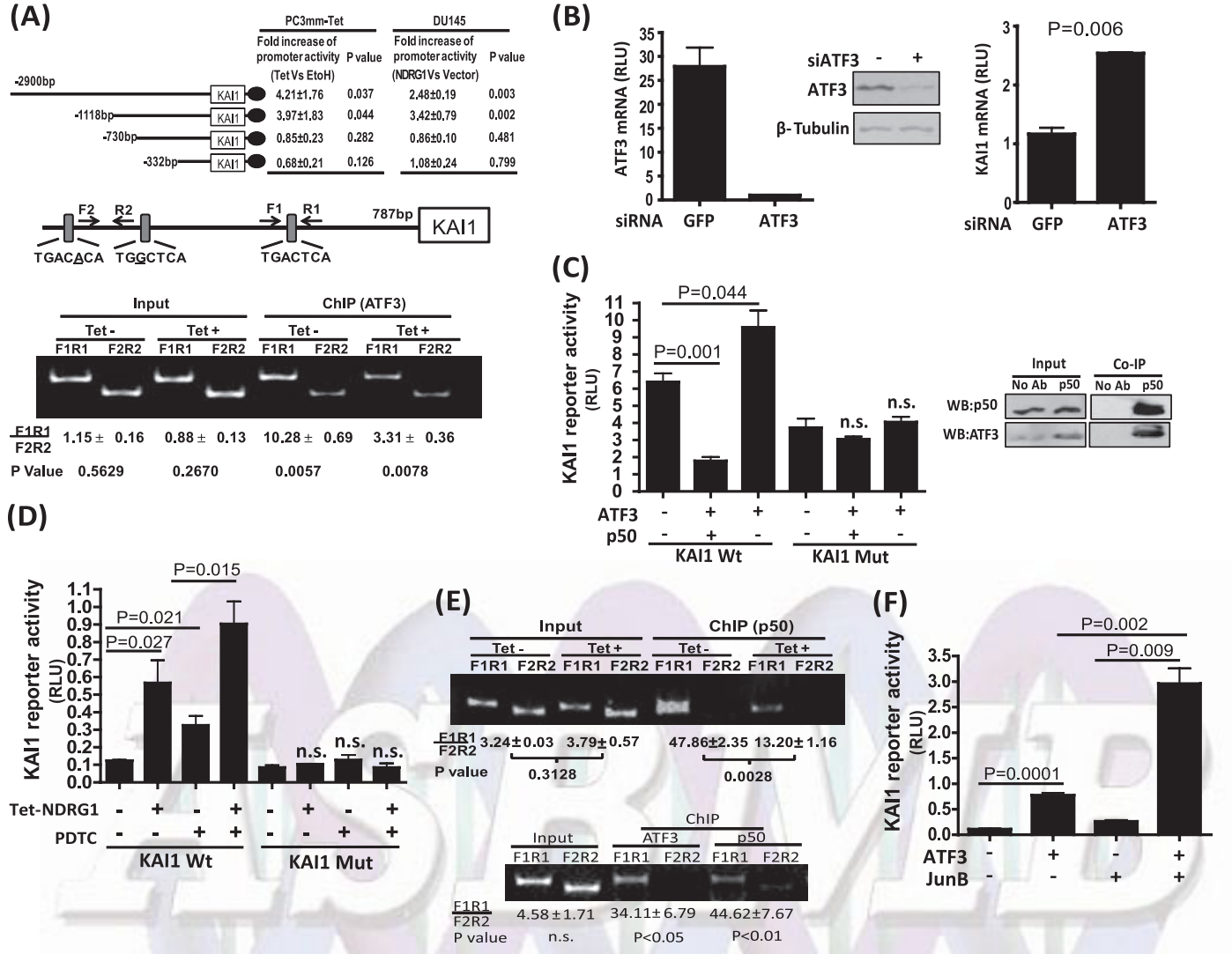

FIGURE 2. **KAI1 expression is regulated through ATF3.** *A*, luciferase reporter constructs with various lengths of the *KAI1* promoter were transfected to PC3mm/Tet-FLAG-NDRG1 and DU145 cells. PC3mm/Tet-FLAG-NDRG1were cultured with or without tetracycline. DU145 cells were transfected with the NDRG1 expression plasmid or an empty vector. Cells were then assayed for luciferase activities (*upper panel*). For ChIP assay (*lower panel*), PC3mm/Tet-FLAG-NDRG1 cells were cultured with or without tetracycline. Precipitated DNA was subjected to quantitative PCR using primers specific for the ATF3 binding consensus sequence on the *KAI1* promoter (F1R1) as well as a control primer set (F2R2). The ratio of the DNA was calculated based on cyclic threshold value for each reaction. *B*, siRNA for ATF3 or GFP was transfected to PC3mm cells, and the expressions of ATF3 (*left*) and KAI1 (*right*) mRNA were examined by quantitative RT-PCR. The expression of ATF3 was also confirmed by Western blot analysis. *C*, *left panel*, wild type or a mutant of *KAI1* luciferase reporter plasmids were transfected into PC3mm cells that were then transfected with expression plasmids of ATF3 and/or p50 and vector control. Cells were then assayed for luciferase activates. *Right panel*, co-immunoprecipitation (*Co-IP*) analysis for the interaction of ATF3 and p50 in PC3mm cells. *D*, wild type or a mutant of *KAI1* luciferase reporter plasmids were transfected into PC3mm/Tet-FLAG-NDRG1 cells that were cultured with or without tetracycline followed by treatment with or without PDTC. Cells were then assayed for luciferase activates. *E*, PC3mm/Tet-FLAG-NDRG1 cells were cultured with or without tetracycline (*upper panel*). RWPE1 cells was infected with Lenti-p50 virus (*lower panel*). Cells were lysed and precipitated with ATFe or p50 antibody. Precipitated DNA was then subjected to quantitative PCR using primers as described above. *F*, *KAI1* luciferase reporter plasmid was transfected into PC3mm cells that were then transfected with expression plasmids of ATF3 and JunB or vector control. Cells were then assayed for luciferase activates. For all experiments, values are means ± S.D. of triplicate measurements. *p* values are based on a two-sided Student's test. *Mut*, mutant.

tional relevance of these two metastasis suppressors in tumor progression. We established permanent cell lines expressing NDRG1 with or without knocking down the *KAI1* gene using the highly metastatic prostate cancer cell line AT6.1 (Fig. 3, *A* and *B*). These cell lines were then examined for their metastatic behavior through a series of functional assays. As we have reported previously, the expression of NDRG1 significantly reduced the invasive ability of prostate cancer cells through Matrigel; however, silencing of the *KAI1* gene significantly abolished NDRG1-induced suppression of invasion (Fig. 3*C*). Moreover, the expression of NDRG1 significantly reduced the migration ability of AT6.1 cells but not in the cells which KAI1expression was knocked down (Fig. 3*D*). Because we have shown that NDRG1 did not affect the growth rate of prostate cancer cells *in vitro* and in primary tumors (33), we examined the effect of NDRG1 on tumor cells grown under anchorage-free conditions. As shown in Fig. 3*E*, we found that the majority of AT6.1 cells (88.9% ± 3.05) maintained their viability during the 72 h of culture in suspension, and NDRG1-overexpressing cells showed significant deficiency in survival under such conditions (66.64% ± 2.07). However, the knockdown of the *KAI1* gene was able to restore anoikis resistance to the same level as

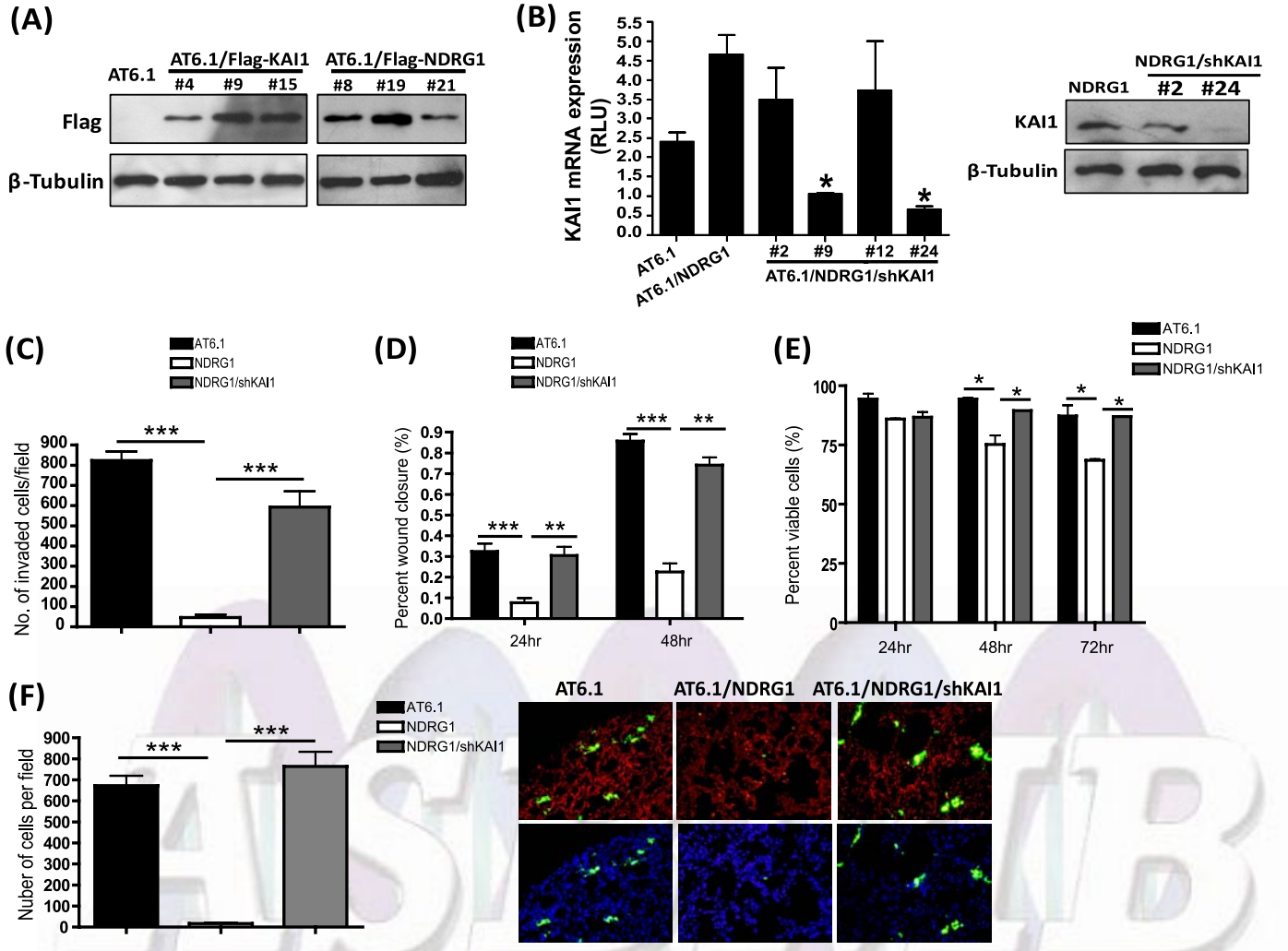

FIGURE 3. **KAI1 is involved in downstream signal of NDRG1-mediated metastasis suppression.** *A* and *B*, establishing Rat prostate cancer cells AT6.1 sublines: AT6.1/NDRG1, AT6.1/KAI1, and AT6.1/NDRG1/shKAI1. Western blot (*A*) confirming overexpression of FLAG-NDRG1 and FLAG-KAI1 in AT6.1/NDRG1 and AT6.1/KAI1 cells. *B*, knockdown of expression of KAI1 was confirmed in AT6.1/NDRG1/shKAI1 cells by RT-PCR (*left*) and (*right*). *C–F*, rat prostate cancer cells, including the parental cell line (AT6.1), NDRG1-transfected sub-lines with (AT6.1/NDRG1/shKAI1) or without (AT6.1/NDRG1) shRNA of KAI1, were subjected to assays for invasion (*C*), migration (*D*), and anoikis resistance (*E*). *F*, AT6.1, AT6.1/NDRG1, and AT6.1/NDRG1/shKAI1 cells were labeled with CellTracker Green and injected into the lateral vein of nude mice ($n = 3$). Lungs were removed after 48 h and sectioned, stained by DAPI, followed by counting the number of tumor cells under the confocal microscope. Lung vasculature was visualized by staining with rhodamine-conjugated lectin. For all experiments, values are means ± S.D. of triplicate measurements. *p* values are based on a two-sided Student's test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

the control (87.72% ± 0.78). Anoikis can be stimulated by detachment from the extracellular matrix in the primary site, which serves as an important barrier to prevent metastatic tumor cell dissemination. Therefore, metastatic tumor cells need to acquire both invasive and anoikis resistance abilities, which aid in thriving during the early stage of metastasis. To directly assess whether KAI1, in conjunction with NDRG1, affects early seeding of tumor cells *in vivo*, we intravenously injected AT6.1 or AT6.1 carrying the expression vector of NDRG1 with or without shRNA to the *KAI1* gene, into nude mice. After 48 h, mice were sacrificed, and the lung sections were examined. We found that the number of NDRG1 expressing cells (17.09 ± 3.69) was significantly lower compared with parental AT6.1 cells (672.2 ± 47.38), whereas KAI1 knockdown (764.1 ± 69.78) substantially abrogated the inhibitory effect of NDRG1 (Fig. 3*F*). Taken together, our results revealed that NDRG1 exerts its metastatic function by up-regulating KAI1, which in turn suppresses various metastatic traits in cancer cells.

NDRG1 and KAI1 Correlate in Clinical Setting and Predict Clinical Outcome of Prostate Cancer—To further examine the relevance of NDRG1 and KAI1 in clinical setting, we performed IHC analysis with tumor specimens from prostate cancer patients ($n = 19$). The results showed that NDRG1 was strongly expressed in the epithelial cells of normal ducts and glands in prostate tissue, whereas it was significantly down-regulated as tumor grades increased ($p = 0.0126$, 0.0003, and 0.0001, respectively, Fig. 4, *A* and *B*). Similarly, KAI1 was also found to be highly expressed in normal prostate epithelium and mainly localized at membrane and cytoplasm, but it was significantly decreased in poorly differentiated tumors ($p = 0.0441$, 0.0051, and 0.0051, respectively, Fig. 4, *A* and *B*). Linear regression analysis revealed a significant positive correlation ($p = 0.0045$) between NDRG1 and KAI1 expression status in prostate cancer

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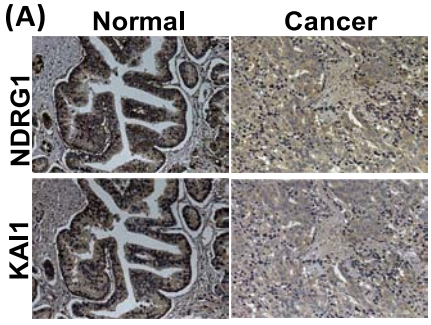
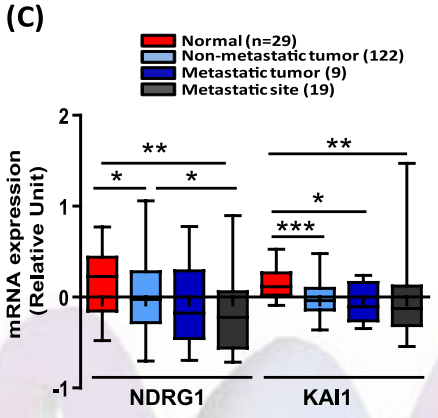
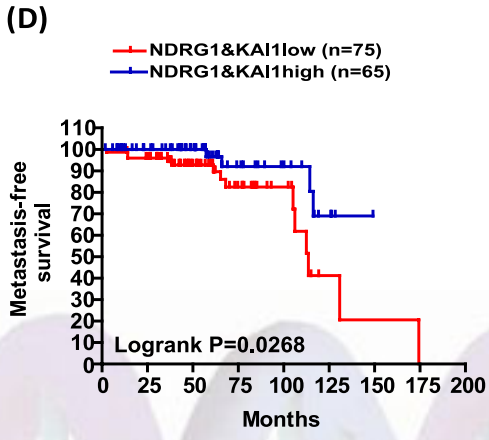


(B)

Expression of NDRG1, in conjuction with KAI1 is down-regualted with advancement of PCa

Gleason grade	No. of lesions	Staining intensity NDRG1	KAI1
2	5	1.9 +/- 0.1	2.39 +/- 0.5
3	15	1.3 +/- 0.1*	1.43 +/- 0.2*
4	13	0.9 +/- 0.1**	0.58 +/- 0.3**
5	5	0.4 +/- 0.1**	0.21 +/- 0.1**





(E)

Multivariate Cox regression for metastasis-free survival

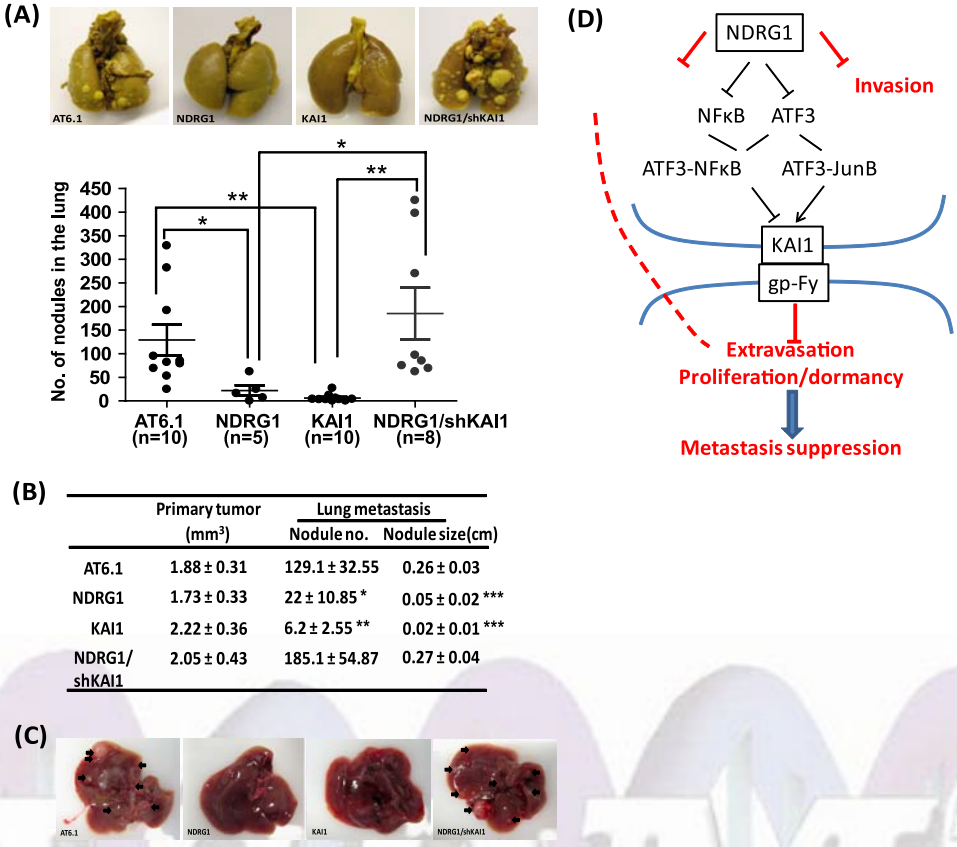
Variable	*P*-value	Hazard ratio (95% CI)
Age at diagnosis (Y) [a]	0.695	0.980 (0.886-1.084)
Lymph nodes [a]	0.064	1.141 (0.992-1.311)
PreDxBxPSA[a]	0.062	1.005 (1.000-1.010)
NDRG1	0.562	1.501(0.380-5.923)
KAI1	0.582	1.390 (0.431-4.484)
Combination of NDRG1 and KAI1	0.046	4.679 (1.030-21.265)

a. Age, lymph nodes and PSA are ordinal variables, treated as continuous, giving hazard ratio per increase in score.

COLOR

FIGURE 4. **KAI1 and NDRG1 are down-regulated in prostate cancer and a combination of two genes expression significantly correlates with patient survival.** *A*, immunohistochemical analysis was performed for NDRG1 and KAI1 with prostate tumor specimens from 19 patients. Representative images for IHC using anti-NDRG1 (*upper panels*) and anti-KAI1 (*lower panels*) antibodies were shown. *B*, staining intensities of both NDRG1 and KAI1 for the specimens were calculated (on a scale of 0–3, with 3 being the highest) for each group of Gleason grade. *C*, expression of *NDRG1* and *KAI1* in tissues from normal, tumor, and distant metastatic sites were examined using the cohort dataset of GSE12034. *p* values are based on a Mann-Whitney Test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *D*, A Kaplan-Meier analysis was done to determine the diagnostic value of the combined expression of *NDRG1* and *KAI1*. The *p* value was determined by log-rank test. *E*, a multivariate Cox regression analysis was conducted to assess the contribution of the indicated variables to disease prognosis.

patients (Fig. 4*B*). Next, we examined the expression of *NDRG1* and *KAI1* in a GEO database that contains the largest number of prostate patient samples to date (accession no. GSE21034). The expression of *NDRG1* and *KAI1* were all significantly down-regulated in tumors and metastatic sites compared with that in normal tissues (Fig. 4*C*). Notably, there was no statistically significant difference between the level of both genes in tissues from metastatic primary tumors and metastatic sites, indicating that the expression of *NDRG1* and *KAI1* are already down-regulated in the primary tumor, which may trigger an activation of metastasis cascade at an early stage. Previously, we have shown that the status of *NDRG1* and *KAI1*, individually, serves as a diagnostic and prognostic marker for disease-free survival of prostate cancer (21, 33). Considering the significant cross-talk between NDRG1 and KAI1 expression in our study, we next evaluated the prognostic value of a combination of these markers. Prostate cancer patients were stratified into two groups based on *NDRG1*/*KAI1* expression and analyzed for their metastasis-free survival by the Kaplan-Meier analysis with a 15-year follow-up. As shown in Fig. 4*D*, patients with a low ($n = 75$) expression level of *NDRG1* and *KAI1* had significantly worse metastasis-free survival than those with a high ($n = 65$) expression (overall log-rank, $p = 0.0268$) of both genes. Importantly, the result of a multivariate Cox regression analysis indicates that the combination of *NDRG1* and *KAI1* was an independent prognostic marker in prostate cancer ($p = 0.046$) with a hazard ratio of 4.679, compared with 1.501 and 1.390 for *NDRG1* and *KAI1* alone, respectively (Fig. 4*E*). These data underscore the utility of the combination of *NDRG1* and *KAI1* as an independent prognostic marker for prostate cancer.



(B)

	Primary tumor (mm^3)	Lung metastasis	
		Nodule no.	Nodule size(cm)
AT6.1	1.88 ± 0.31	129.1 ± 32.55	0.26 ± 0.03
NDRG1	1.73 ± 0.33	22 ± 10.85 *	0.05 ± 0.02 ***
KAI1	2.22 ± 0.36	6.2 ± 2.55 **	0.02 ± 0.01 ***
NDRG1/ shKAI1	2.05 ± 0.43	185.1 ± 54.87	0.27 ± 0.04

(C)

FIGURE 5. **KAI1 expression is indispensible for NDRG1-mediated metastasis suppression in animal.** Rat prostate cancer cells including the parental cell line (AT6.1), NDRG1-transfected sub-lines with (AT6.1/NDRG1/shKAI1) or without (AT6.1/NDRG1) shRNA of KAI1, and KAI1-transfected sub-line were injected subcutaneously into SCID mice. After 30 days, the mice were sacrificed, and lungs were removed. Tumor nodules on the lungs were counted macroscopically. *A*, *upper panels* show representative images of lung metastases. The lower panel shows the number of tumor-bearing mice and nodules on the lungs. Sizes of primary tumor and nodules on the lungs are shown in *B*. Data are presented as the mean nodule number or size. *Error bars* represent the S.E. *p* values are based on a two-sided Student's test. *, $p < 0.05$; **, $p < 0.01$. *C*, representative images of liver metastases. *D*, concerted activities of NDRG1 and KAI1-mediated metastasis suppression.

KAI1 Knockdown Abrogated Suppressive Effect of NDRG1 on Metastasis in Animals—To further verify our notion that NDRG1 suppresses metastasis through activation of KAI1 *in vivo*, the metastatic cell line AT6.1 and its derivatives, AT6.1/NDRG1, AT6.1/KAI1, and AT6.1/NDRG1/shKAI1, were individually inoculated subcutaneously into the dorsal flanks of SCID mice. The mice were monitored for the formation of primary tumors and were sacrificed after 30 days. The lungs were then removed, and the number of metastatic lesions was grossly counted. We found that all cell lines formed a primary tumor with similar size (Fig. 5*B*), confirming the notion that *NDRG1* and *KAI1* genes do not have an apparent effect on tumorigenesis. However, cells with overexpressing the *NDRG1* or *KAI1* gene showed a significantly lower lung metastases compared with the parental cell line AT6.1. As shown in Fig. 5, *A* and *B*, AT6.1/NDRG1 and AT6.1/KAI1 cells generated not only a significantly fewer number of lesions ($p = 0.042$, and 0.0014, respectively) but also a smaller size ($p < 0.0001$ and 0.0001, respectively) of nodules in the lungs, suggesting that NDRG1 or KAI1 alone can functionally attenuate the seeding and colonization abilities of tumor cells. Importantly, knockdown of KAI1 expression in NDRG1-overexpressing cells almost completely abrogated the metastasis suppressive ability of the *NDRG1* gene, which is consistent with our observation in *in vitro* studies. Interestingly, we found that mice (two of ten) inoculated with AT6.1 parental cells developed extensive liver metastasis in addition to lung metastasis, whereas none of the mice that received AT6.1 carrying the NDRG1- or KAI1-overexpressing vector developed liver metastasis. On the other hand, knockdown of KAI1 expression restored the ability of AT6.1 to develop liver metastasis. Therefore, KAI1 serves as an essential downstream factor of NDRG1-mediated metastasis suppression in our animal model.

DISCUSSION

In this report, we examined the regulatory network of two metastasis suppressor genes, *NDRG1* and *KAI1*, and demonstrated that NDRG1 up-regulated KAI1 expression through modulation of ATF3 signaling in prostate cancer cells. Our results also indicate that KAI1 is a critical downstream target in the NDRG1 pathway and that loss of KAI1 expression significantly abrogates NDRG1-mediated metastatic suppression *in vitro* and *in vivo*. The concerted metastasis suppressor activity of NDRG1 and KAI1 was also evidenced by the result of clinical analysis that a combined expression status of these two genes serves as an independent diagnostic marker to predict metastasis-free survival of prostate cancer patients.

Down-regulated KAI1 expression has been observed in a variety of cancers; however, genetic alterations such as loss of heterozygosity and a mutation within the coding region or epigenetic modification of this gene is a rare event (16–20). Rather, it is likely that the replacement of transcriptional activators for the *KAI1* promoter by the dominant co-repressor complex serves as a major mechanism to repress KAI1 expression (43). The *KAI1* promoter region contains putative binding motifs for various transcription factors. The NFκB p50 subunit was found to bind the region between −6631 and −6996 bp upstream to the *KAI1* gene transcription start site (23, 24). However, several lines of evidence indicate that NFκB alone does not affect KAI1 transcription. Kim *et al.* (25) demonstrated recently that β-catenin in combination with the Reptin chromatin remodeling complex replaced the Tip60-Pontin co-activator complex on the *KAI1* promoter, where the p50 homodimer serves as a dock for both the β-catenin-Reptin and Tip60-Pontin complex during this binding complex switching process. In another study in determining a role of IκB kinase α (IKKα, an NFκB signaling activator) in prostate cancer metastasis, Luo *et al.* (44) generated a variant of TRAMP mice with IKKα deficiency (TRAMP $IKK\alpha^{AA/AA}$) and prevented activation of NFκB signaling. They found that WT/TRAMP mice developed prostate cancer and metastasis significantly earlier compared with TRAMP $IKK\alpha^{AA/AA}$; however, KAI1 expression was not different in both mice, thus strongly indicating that NFκB alone does not regulate KAI1 transcription.

Marreiros *et al.* (27, 28) previously identified a region of the KAI1 promoter that contained binding motifs for AP-2 (α, β, or γ), p53, and AP-1 (Jun and Fos) that is essential for a high level promoter activity in bladder and prostate cancer cells. Detailed examination of KAI1 promoter activity in a series of prostate cancer cells with low to high malignancy showed the requirement of three different transcription factors for the maximum promoter activity, whereas decrease or loss of functional p53 in conjunction with differential levels of AP-1 and AP-2 proteins in highly malignant cells frequently led to formation of a co-repressor complex to suppress *KAI1* promoter activity. In our study, we showed that deletion of a similar region in the *KAI1* promoter containing AP-2, p53, and AP-1 sites significantly abolished the NDRG-induced activation of the *KAI1* promoter in PC3mm and DU145 prostate cells. Because both cell lines are absent of p53 function, our observation is likely to be independent of p53 activity, and it is rather considered to be a result of an interplay between other transcription factors. Of note, we found that knockdown of ATF3 significantly enhanced the *KAI1* mRNA level; however, in contrast to our expectations, ectopic expression of ATF3 increased *KAI1* promoter activity. We attributed this apparent discrepancy to the effect of differential experimental settings of silencing the endogenous expression and forced expression of ATF3, which may substantially alter cellular endogenous environment. We propose that silencing ATF3 expression directly affects the ATF3 co-repressor complex such as ATF3-p50 on the *KAI1* promoter, thus releasing *KAI1* from such suppressive signals. On the other hand, forced expression of ATF3 forms a co-activator complex with endogenous AP-1 proteins JunB (existing in PC3mm cells, see Ref. 27) and promotes *KAI1* transcription activity. We have indeed shown that co-transfection of p50 and ATF3 tilted the balance of endogenous transcription factors and led to formation of ATF3-p50 co-repressor complex, which significantly suppressed *KAI1* promoter activity.

It is noteworthy that, in our analysis for prostate cancer cohort data (GEO accession no. GSE21034), expression of the *NFKB1* gene was found to be increasingly elevated with clinical grade becoming higher. Interestingly, we also found that the *JunB* expression was maintained at a relatively high level in low grade tumors, and it substantially decreased in high grade lesions (data not shown). Consistently, Konishi *et al.* (45) examined the expression status of JunB by IHC analysis and found that JunB expression was decreased significantly in high grade lesions with Gleason score ≥6 and in metastatic lesions. A more recent study revealed that JunB was a Smad1-responsive gene and that it was involved in suppression of prostate cancer metastasis (46). On the other hand, the role of NFκB transcription factors has been well established in the development and progression of prostate cancer through its effect on apoptosis, invasion, and inflammation and particularly on androgen-independent and recurrent prostate cancer (47–50). Therefore, ATF3 acts as a modulator of *KAI1* transcription through coordinating other endogenous transcription factors as either co-activator or co-repressor, and this mechanism is dependent on the tumor stage and genetic background of tumors.

It is well documented that the expressions of metastasis suppressors were generally lost during tumor progression; however, a detailed mechanism of their loss is yet to be elucidated, and many critical questions remain unanswered. When does the expression of these metastasis suppressors start to diminish? Is it a sequential or dynamic process? Does loss of expression of one factor affect others and is there any functional complementary mechanism existing? In our *in vitro* experiment, we clearly showed that ectopic expression of NDRG1 induced up-regulation of KAI1 by indirectly inhibiting the transcription factor ATF3. Interestingly, previous studies reported that NDRG1attenuated the nuclear translocation of NFκB and its binding to the NFκB binding motif by suppressing the expression of the κB kinase inhibitor IKKβ and also by blocking IκBα phosphorylation (38). It should be noted that the 5′-UTR region of the ATF3 gene contains the NFκB motif, which is involved in the signaling of stress stimuli (54), suggesting that NDRG1 inhibits ATF3 expression through modulation of NFκB. Therefore, a high level of NDRG1 expression seems to relieve KAI1 from the inhibitory signal of both ATF3 and NFκB. We also examined the relevance of NDRG1 and KAI1 in a broad range of prostate and breast cancer cell lines and in clinical samples, and our results strongly indicate that NDRG1 and KAI1 expression are correlated significantly to each other. Interestingly, we also found that down-regulation of both NDRG1 and KAI1 already took place in the primary tumor in patients without metastasis, although it will be more obvious in metastatic patients. Consistently, El Touny *et al.* (51) previously discovered an age-dependent down-regulation of KAI1 in the TRAMP mice model, which started from 24 weeks when the tumor developed but before metastasis occurred. We also found that *Ndrg1* was down-regulated significantly in the hyperplasia stage of MMTV-*Wnt1* and MMTV-*neu* transgenic

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mice (supplemental figure). Taken together, these results indicate that the loss of expression of both NDRG1 and KAI1 occurs in parallel, and it is considered to take place at a relatively early stage. Notably, we have shown that KAI1 is an essential downstream factor of NDRG1 signaling in our *in vitro* and animal experiments. Knocking down the expression of KAI1 not only abrogated NDRG1-mediated motility/invasion suppression of tumor cells but also restricted early seeding and later colonization of metastatic tumor cells in distant sites. Importantly, our results also suggest that the combination of *NDRG1* and *KAI1* expression status has a strong independent value for predicting patient outcome compared with either marker alone. Our results demonstrate that KAI1 is a downstream target and an effector of NDRG1, suggesting the concerted regulatory mechanism and activity of these metastasis suppressor genes. We also have shown that a small compound, Dp44mT, which activates the NDRG1 gene, significantly enhanced KAI1 expression. Considering that functionally reconstituting metastasis suppressor genes could be beneficial to clinical treatment of metastatic disease, our results provide a novel insight to develop strategies for metastasis suppressor-based therapies.

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Review

Metabolic genes in cancer: Their roles in tumor progression and clinical implications

Eiji Furuta, Hiroshi Okuda, Aya Kobayashi, Kounosuke Watabe *

Department of Medical Microbiology and Immunology, Southern Illinois University School of Medicine, Springfield, Illinois, USA

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ABSTRACT

Re programming of metabolic pathways is a hallmark of physiological changes in cancer cells. The expression of certain genes that directly control the rate of key metabolic pathways including glycolysis, lipogenesis and nucleotide synthesis are drastically altered at different stages of tumor progression. These alterations are generally considered as an adaptation of tumor cells; however, they also contribute to the progression of tumor cells to become more aggressive phenotypes. This review summarizes the recent information about the mechanistic link of these genes to oncogenesis and their potential utility as diagnostic markers as well as for therapeutic targets. We particularly focus on three groups of genes; GLUT1, G6PD, TKTL1 and PGI/AMF in glycolytic pathway, ACLY, ACC1 and FAS in lipogenesis and RRM2, p53R2 and TYMS for nucleotide synthesis. All these genes are highly up regulated in a variety of tumor cells in cancer patients, and they play active roles in tumor progression rather than expressing merely as a consequence of phenotypic change of the cancer cells. Molecular dissection of their orchestrated networks and understanding the exact mechanism of their expression will provide a window of opportunity to target these genes for specific cancer therapy. We also reviewed existing database of gene microarray to validate the utility of these genes for cancer diagnosis.

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* Corresponding author. Department of Medical Microbiology and Immunology, Southern Illinois University School of Medicine, 801 N. Rutledge St., P.O. Box 19626, Springfield, Illinois 62794-9626, USA. Tel.: +1 217 545 3969; fax: +1 217 545 3227.
E-mail address: kwatabe@siumed.edu (K. Watabe).

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1. Introduction

1.1. Metabolic changes in cancer

Energy homeostasis of a normal cell is balanced by at least three metabolic pathways including glycolysis, lipogenesis and tricarboxylic acid (TCA) cycle, and these pathways are also closely linked to amino acid as well as nucleotide biosynthesis. Although normal cells utilize a variety of energy sources such as glycogen, fatty acids and amino acid, glucose is considered as a key energy source for their growth. Glucose is taken up by the glucose transporter system and is converted to pyruvate through the glycolysis pathway [1]. Pyruvate is then converted to acetyl CoA and utilized as a substrate for the TCA cycle in mitochondria. While the TCA cycle generates ATP through its oxidative phosphorylation, an intermediary metabolite, citrate, is exported into the cytoplasm and is converted to acetyl CoA which is used as an initial substrate for generating fatty acids through the lipogenesis pathway [2,3]. Fatty acids serve not only as energy storage but also provide key components for membrane biosynthesis and also play important roles in cell signaling by modifying various signal proteins (Fig. 1).

On the contrary to the normal cells, tumor cells exhibit quite abnormal behavior by re programming these metabolic pathways. It has long been recognized that the cancer cells need to drive higher rate of energy metabolism because of their active proliferation rate and motile nature. With the same reasons, tumors become more hypoxic and therefore they need to rely on nonoxidative energy source such as glycolysis as originally reported by Warburg [4]. On the other hand, higher rate of lipogenesis in cancer cells seems to be contributing both to building mass (cell membrane etc) and gen erating energy (beta oxidation) [5]. The rate of lipogenesis is also significantly accelerated in tumor cells in order to compensate for the higher rate of proliferation. This metabolic re programming triggers a series of cascade of events in tumor cell physiology and often gen erates harmful byproducts such as ROS and sometimes even im balance nucleotide pool for DNA replication that promote a mutation rate through the activation of mutators and/or the defect of DNA repair system. It was also shown that the germline mutations were significantly correlated with the incidence of various types of cancer in clinical setting [6 8]. The major source of ROS in normal cells is oxidative phosphorylation; however, ROS is also generated by other pathways such as NADH oxidation. In fact, many cancer cells have high turn over of glycolysis and promote the NADH oxidative pathway which consequently generates high amount of ROS. On the other hand, it is known that many cancer cells have malfunctioning mitochondria which results in lower oxidative phosphorylation. However, this is not always the case and recent evidence demon strated that many cancer cells still have intact mitochondria with normal function of TCA cycle.

It is increasingly evident that many genes involved in metabolic pathways play direct roles in tumorigenesis and tumor progression. In the following sections, we review the most current information about these metabolic genes in regard to their physiological roles in tumorigenesis and underlining molecular mechanisms. We also discuss their possible utilities as diagnostic markers and potential therapeutic implications.

2. Glucose metabolism in cancer

2.1. Cancer cells depend on glycolysis for energy supply

In most cancer cells, the rate of glucose uptake is significantly elevated and oxidative phosphorylation in mitochondria is often decreased compared to normal cells. This effect was first noted by Otto Warburg in 1929 and is called as aerobic glycolysis or the Warburg effect [4]. Rapidly growing cancer cells suffer from a lack of oxygen and nutrition due to the diffusion limits of blood supply, and therefore, persistent glucose metabolism and generation of lactate is thought to be an adaptation of tumor cells to hypoxia. Interestingly, however, cancer cells prefer to utilize glycolysis for their energy supply even under normoxic condition when they are grown in culture medium. Although glycolysis is far less efficient in generating ATP than oxidative TCA cycle, it is much faster than the oxidative pathway and is independent of mitochondrial function which is often dysfunctional in cancer cells. In fact, Ramanathan et al. showed that even when mitochondrial function was completely blocked, i.e., inhibition of oxidative ATP production, the level of ATP was not significantly altered in tumor cells [9]. However, how cancer cells reprogram this metabolic alteration and whether it is essential for tumorigenesis is still not fully understood. One key factor which links glycolysis and tumorigenesis is the tumor suppressor, p53. This gene appears to block glycolytic pathway through its target TIGER (TP 53

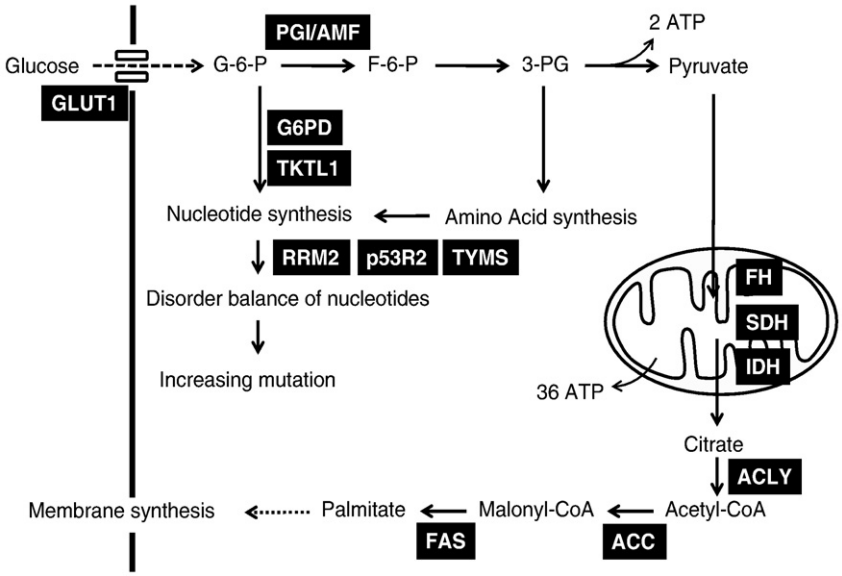

Fig. 1. Metabolic genes in cancer. GLUT1; glucose transporter 1, G6PD; Glucose-6-phosphate dehydrogenase, TKTL1; transketolase-like-1, RRM2; ribonucleotide reductase subunit M2, p53R2; p53-inducible ribonucleotide reductase small subunit 2 homolog, TYMS; thymidylate synthase, FH; fumarate hydratase, SDH; succinate dehydrogenase, IDH; isocitrate dehydrogenase, ACLY; ATP citrate lyase, ACC; acetyl-CoA carboxylase, and FAS; fatty acid synthase.

induced glycolysis and apoptosis regulator) by decreasing the glycolytic metabolite fructose 2,6 bis phosphate which stimulates glycolysis and inhibits gluconeogenesis [10]. p53 has also been shown to down regulate another glycolytic enzyme, phosphoglycerate mutase (PGM). Furthermore, EGFR which is highly expressed in many cancers has been shown to inhibit autophagic cell death by maintaining intracellular glucose level through stabilization of the sodium/glucose cotransporter 1 (SGLT1) [11]. In addition, oncogenes such as Ras and Src have been reported to promote glycolysis by activating glucose transporter (GLUT) 1 which is a key gene of glucose uptake [12,13]. Therefore, re programming the glycolytic pathway is considered to play critical roles in tumorigenesis and tumor progression. Currently, there are at least four genes in the glycolytic pathway that are known to be directly involved in oncogenesis, namely GLUT1, PGI/AMF, G6PD and TKTL1 (Table 1, [14 22]). The product of GLUT1 is capable of transporting glucose across the hydrophobic cell membrane, which is the first rate limiting step of glucose metabolism. The up regulation of GLUT1 and GLUT3 with increased glucose uptake has been shown in various tumors including oesophageal, gastric, breast and colon cancers [62 65]. Phosphoglucose isomerase/autocrine motility factor (PGI/AMF) catalyzes the second glycolytic step, the isomerization of glucose 6 phosphate to fructose 6 phosphate [66]. Recent studies revealed that PGI/AMF is a multifunctional moonlighting protein which is associated with not only glycolysis but also cancer cell migration, invasion, growth, survival, and angiogenesis. Glucose 6 phosphate dehydrogenase (G6PD) and transketolase like 1 (TKTL1) are involved in an important branch of glycolysis, pentose phosphate pathway. Both G6PD and TKTL1 are key enzymes for ribose production, and therefore, they are considered to play roles in tumor cell proliferation [17,67].

2.2. GLUT1 mediates glucose uptake in cancer cells

Glucose is a polar molecule and cannot penetrate endothelial cells or plasma membrane by simple diffusion, and therefore, uptake of glucose across cell membranes requires transporter proteins. GLUT 1, GLUT3, and GLUT4 are members of GLUT/SLC2 family and they are known to regulate glucose uptake [68,69]. The GLUT family is a transmembrane protein which has 12 transmembrane domains with both amino and carboxy terminal ends exposed to the cytoplasmic side of the plasma membrane. GLUT1 is found at variable levels in many tissues, while GLUT3 and GLUT4 are expressed in a tissue specific manner [65,70]. Increased expression of GLUT1 has been shown in various types of cancers including hepatic, pancreatic, breast, esophageal, brain, renal, lung, cutaneous, colorectal, endometrial, ovarian and cervical carcinoma [71 80]. Notably, high expression of GLUT1 is significantly correlated with decreased survival in breast cancer. The expression of GLUT1 and glucose uptake was also strongly increased in rat renal oncocytic tubules when renal oncocytomas were induced by chemicals [81]. These data suggest that GLUT1 acts as an oncogene in a variety of cancers. In fact, ectopic expression of GLUT1 in Chinese hamster ovary cells led to a higher rate of glucose and thymidine uptake when cells were exposed to glucose deficient conditions, indicating that GLUT1 support tumor cell growth [82]. Increased GLUT1 expression and glucose uptake enables rapidly growing cancer cells to acquire energy even under hypoxic condition by harnessing glycolysis. Of note, hypoxia inducible factor (HIF) which is up regulated in many cancers is known to enhance the expression of GLUT1 and other enzymes that are necessary for glycolysis [83,84]. In addition, HIF 1 promotes angiogenesis through up regulation of VEGF, which facilitates intake of oxygen as well as glucose by tumor cells [85]. Interestingly, HIF 1 is induced not only by hypoxia but also by Ras through PI3K/Akt, resulting in VEGF up regulation [86]. Therefore, GLUT1 plays an important role in the proliferation of cancer cell by supplying energy source, and the expression of this gene is critically balanced by various oncogenes and tumor microenvironment.

The phenomenon of elevated glucose uptake has been clinically exploited to detect tumor cells by positron emission tomography (PET) scan using the glucose analogue tracer 2 fluorodeoxy D glucose

Table 1
Metabolic genes in cancer.

	Function of gene	Up-regulation in cancer (IHC)	Cell growth (*in vitro*)		Xenograft tumor	Tumor in TG mouse	Secretion	Inhibitor	Reference
			Ectopic expression	Inhibition					
Glucose metabolism									
GLUT1	Glucose transporter	Lung, colon, ovary, bile duct	Promote	–	Promote	–	–	2DG	[14–16]
G6PD	Glucose-6-phosphate dehydrogenase	Lung, breast, colon, prostate	Promote	–	Promote	–	–	6-AN, BSO	[17,18]
TKTL1	Transketolase	Colon, ovary, stomach, urothelium	Promote	Arrest	–	–	–	siRNA, oxythiamine chloride	[19,20]
PGI/AMF	Glucose phosphate isomerase/ autocrine motility factor	Lung, breast, bone, stomach	Promote	Arrest	Promote	Promote	Serum, urine	siRNA, E4P, M6P	[21,22]
Lipid metabolism									
ACLY	ATP citrate lyase	Lung	–	Arrest	Promote	–	–	siRNA, SB-204990	[23–25]
ACC1	Acetyl-CoA carboxylase	Lung, breast	–	Arrest	–	–	–	siRNA, TOFA	[26–28]
FAS	Fatty acid synthase	Lung, breast prostate, colon	Promote	Arrest	Promote	Promote	Serum	Cerulanin, Orlistat, C75, C93	[29–46]
Nucleotide metabolism									
RRM2	Ribonucleotide reductase	Lung	–	Arrest	Promote	Promote	–	siRNA, GTI-2040	[28,47–50]
p53R2	Ribonucleotide reductase	Lung	Promote	Arrest	–	Promote	–	siRNA	[47,51–53]
TYMS	Thymidylate synthase	Lung, breast, colon, cervical	Promote	Arrest	Promote	Promote	–	siRNA, ZD1694, 5-FU	[54–57]
Mitochondrial metabolism									
SDHB,C,D	Succinate dehydrogenase	Neuroglia, adrenal gland	–	Promote[a]	Promote[a]	–	–	siRNA	[58,59]
Fh1	Fumarate hydratase	Smooth muscle, kidney	–	ND	ND	–	–	siRNA	[60]
IDH1	Isocitrate dehydrogenase	Breast, prostate, neuroglia, brain	Promote[b]	–	–	–	–	siRNA, oxalomalate	[61]

ND; No difference.

[a] Tumor growth was measured in athymic nude mice implanted 143B human osteosarcoma or 143B SdhB shRNA clone. Inhibition of SdhB promoted the tumor growth *in vitro* and *in vivo*.

[b] Ectopic expression of IDHR132H mutant in U-87 MG cells elevated HIF-1 alpha expression which promoted tumor growth.

(2FDG) [87,88]. Importantly, the dependence of cancer cells on glycolysis can also be utilized to selectively inhibit cancer cells in chemotherapy. 2DG is a glucose analogue and is converted to 2DG 6 phosphate which inhibits glycolytic enzymes, phosphoglucose isomerase and hexokinase. Consequently, 2DG treated cell cannot effectively use glucose as energy source, which results in the energy deprivation and the following growth arrest of tumor cell [89]. These results suggest a potential utility of 2DG as anti cancer drug, although 2DG is known to cause hypoglycemic symptoms because it also reduces glucose in normal tissues, especially in the brain which heavily relies on glycolysis for energy supply [90,91]. A recently performed phase I clinical trial (NCT00096707) for 2DG treatment on several types of cancer patients showed that 2DG exhibited positive responses in patients who were treated orally with this compound. These data support our continuing hope that 2DG serves as a lead compound to develop a better drug to target glycolytic pathway for cancer therapy. In addition, the results of phase I/II clinical trials have shown that the combination of 2DG and γ radiation was well tolerated in cerebral glioma patients [65]. Therefore, such combination therapies by taking advantage of the dependence of cancer cells on glycolysis may also be a promising approach for cancer treatment.

2.3. G6PD is a key enzyme of pentose phosphate pathway

G6PD is a key enzyme to produce ribose 5 phosphate via pentose phosphate pathway, which is essential for RNA and DNA synthesis in rapidly growing cells [17,67]. Another crucial role of G6PD is to generate NADPH which is an essential factor for glycolysis, and the reducing power of NADPH is necessary to neutralize oxidative stress, e.g., to maintain the reduced form of glutathione which serves to detoxify free radicals and peroxides [92]. Therefore, G6PD is thought to contribute to cancer growth and survival by producing ribose and NADPH through pentose phosphate pathway. In fact, elevated levels of expression and activity of G6PD are frequently observed in breast, colon, endometrial, cervical, prostatic, and lung cancers [93,94]. Interestingly, ectopic expression of G6PD in NIH 3T3 cells was shown to significantly increase intracellular levels of NADPH and glutathione and also to promote anchorage independent cell growth [17,92]. Furthermore, these cells were shown to be tumorigenic as well as angiogenic in nude mice, suggesting that G6PD acts as oncogene.

The higher rate of glycolysis in cancer cells generates increased number of metabolites such as hydrogen ions and lactate that cause acidification of the cells. Although acidosis can stimulate invasion, migration, mutagenesis, and radioresistance in cancer cells, it also causes apoptosis through the p53 pathway [1,95]. Elevated glycolytic flux via HIF1 causes lactate production by up regulation of lactate dehydrogenase to increase pyruvate to lactate flux [96,97] and also by pyruvate dehydrogenase kinase (PDK) to block pyruvate recruitment into the tricarboxylic acid cycle [98,99]. To avoid resultant acidosis and maintain an intracellular pH, cancer cells need to pump out H+ ion by utilizing Na+/H+ antiporter or by monocarboxylate transporter which transports H+ with lactate [100,101]. Considering the fact that ectopic G6PD expression increases the level of NADPH and glutathione, G6PD may contribute to cancer cell survival by maintaining the intracellular pH and redox balance.

Due to the critical role of G6PD in tumorigenesis, this enzyme is considered to be an excellent therapeutic target. Buthionine S′R′ sulfoximine (BSO), a glutathione depletion agent, is known to inhibit G6PD, and this compound was shown to suppress colony formation of G6PD expressing cells in soft agar [17]. BSO is currently in phase I clinical trial (NCT00006027, NCT00002706, NCT01007305, NCT00002706, NCT00002730, NCT00005835 and NCT00661336). Another promising inhibitor of G6PD is 6 aminonicotinamide (6 AN) which has been used as a modulator of smooth muscle contraction [18]. 6 AN is also capable of suppressing pentose phosphate pathway by inhibiting 6 phosphogluconate dehydrogenase which results in NADPH reduction, suggesting that this compound can be an effective inhibitor of pentose phosphate pathway. Furthermore, combination of 2DG and 6 AN has been shown to enhance the radiosensitivity in human glioma and squamous carcinoma cell lines [102]. Therefore, a combination of inhibitors for G6PD and glucose transporter can be an effective approach to selectively suppress cancer cell growth.

2.4. Transketolase like 1 supports tumor proliferation through pentose phosphate pathway

Thiamine (vitamin B1) dependent transketolase is another key enzyme of pentose phosphate pathway. Transketolase regulates the nonoxidative pathway of pentose phosphate pathway, while G6PD is responsible for the oxidative pathway. Like G6PD, TKTL1 gene was also shown to be strongly expressed in various carcinomas including ovarian, nasopharyngeal, colon and urothelial carcinomas [103 105]. Increased expression of TKTL1 is correlated with higher tumor stages, invasion, and poor prognosis [104,106]. Importantly, inhibition of TKTL1 by RNAi in nasopharyngeal carcinoma cell line (CNE) dramatically down regulated transketolase activity and significantly inhibited proliferation of the cells [19]. In addition, knock down of the expression of TKTL1 by siRNA in colon cancer cell line (LoVo) was accompanied with decreased proliferation and G0/G1 arrest [107]. Furthermore, TKTL1 knock down sensitized colon carcinoma cells (HCT116) to oxidative stress induced apoptosis [108]. On the other hand, induction of TKTL1 by thiamine promotes cell growth in Ehrlich's ascites tumor cells [103]. Of note, thiamine is metabolized to thiamine pyrophosphate, a cofactor of transketolase, which is involved in ribose synthesis, and promotes cell replication. Therefore, TKTL1 contributes to tumor progression by promoting cell survival and also by providing ribose via pentose phosphate pathway for tumor cell growth. To date, a specific inhibitor for TKTL1 has not been identified [20]; however, this enzyme is considered to be a rational target for cancer therapy.

2.5. PGI/AMF has dual roles as a glycolytic enzyme and a cytokine

PGI was originally isolated as “autocrine motility factor (AMF)” from the conditioned medium of human A2058 melanoma cells. As a tumor secreted cytokine, PGI/AMF has been shown to be involved in cell migration, invasion, proliferation, survival, and angiogenesis [109]. Interestingly, PGI/AMF plays another important role in glycolysis and gluconeogenesis and this enzyme catalyzes the second glycolytic step, the isomerization of glucose 6 phosphate to fructose 6 phosphate [22]. Cell surface receptor of PGI/AMF, gp78/AMFR, is overexpressed in various metastatic tumors along with PGI/AMF, and their presence in the serum and urine is correlated with a poor prognosis and tumor progression [21,110 117]. Ectopic expression of PGI/AMF in murine fibroblasts and fibrosarcomas rendered the cells highly motile and transformed phenotype *in vitro* and tumorigenicity *in vivo* [118,119]. Furthermore, orthotopic implantation of pancreatic tumor cells that ectopically expressed PGI/AMF produced local tumors and liver metastases [120]. On the other hand, the suppression of PGI/AMF expression led to the inhibition of cell proliferation and tumorigenicity followed by mesenchymal to epithelial transition [121]. Furthermore, down regulation of PGI/AMF in mouse embryonic fibroblasts and human fibrosarcoma caused premature senescence which is regulated in part by tumor suppressor genes [21,122]. It has been shown that the tumor suppressor p53 down regulated PGI/AMF and that cyclin dependent kinase inhibitor p21 was increased in PGI/AMF knock down cells, suggesting that inhibition of PGI/AMF may be an effective way to suppress tumor cells by inducing senescence [21]. Erythrose 4 phosphate (E4P) and mannose 6 phosphate (carbohydrate phosphates) are known to specifically inhibit PGI/AMF and considered to induce senescence to tumor cells [22]. Therefore, these compounds and their analogues may potentially

serve as effective anti cancer drugs. Interestingly, the level of PGI/AMF in urine has been shown to be increased in patients with transitional cell carcinoma of bladder, therefore urinary PGI/AMF may be a useful marker for diagnosis of bladder cell carcinoma [111].

3. Lipogenesis and cancer

3.1. Lipogenic pathway is activated in cancer cells

Re programming of lipogenic pathway is one of the most significant alterations of tumor cell physiology and at least three genes in this pathway are known to play key roles in tumor progression, namely ACLY, ACC and FAS. Triacylglycerol is an esterified form of glycerol which consists of three fatty acids including palmitate, oleic acid and alpha linolenic acid and it is stored mostly in hepatic and adipose cells to maintain energy homeostasis. As a first step of fatty acid synthesis, pyruvate needs to be converted to acetyl CoA in the mitochondria. Acetyl CoA is then incorporated into TCA cycle which produces citrate in the presence of sufficient amount of ATP. Accumulated citrate is exported to the cytoplasm where it is catalyzed by ATP citrate lyase (ACLY) to generate cytosolic acetyl CoA which is a key precursor of fatty acids. Acetyl CoA is then carboxylated by ACC to synthesize malonyl CoA which is then converted to palmitate (16 carbon saturated fatty acid) as the first fatty acid in lipogenesis by the key rate limiting enzyme [123]. ACC and FAS are both highly expressed in the embryonic cell and the functions of both enzymes are essential for development. In fact, mice deficient in these enzymes died at embryonic stage [124,125]. On the other hand, it is well recognized that fatty acid synthesis pathway is significantly activated at a relatively early stage in various types of tumors, and the key genes involved in this pathway including ACLY, ACC and FAS are considered to play critical roles in tumorigenesis and cancer progression (Table 1, [23 46]). In this section, we will discuss the functional roles of these three genes in tumor progression and potential therapeutic as well as diagnostic implications.

3.2. ATP citrate lyase generates cytosolic acetyl CoA for lipid synthesis

Citrate is generated by citrate synthase in the TCA cycle and is exported to the cytosol through mitochondrial citrate transporter. It is then converted by ACLY to cytosolic acetyl CoA which serves as an essential component for fatty acid synthesis. While the expression of ACLY is low in normal cells, it is significantly up regulated in various types of tumors [126 130]. Of note, phosphorylated ACLY (active form of ACLY) was found to be positively correlated with clinical stages of lung cancer [23]. Furthermore, ACLY inhibitors such as siRNA and SB 204990 block the production of acetyl CoA and consequently suppress cell growth *in vitro* and *in vivo* [24,25]. Blocking ACLY with siRNA causes the suppression of Akt signaling and thus results in the loss of tumorigenicity *in vitro*. These results indicate that ACLY plays a role in tumorigenesis and tumor cell survival and suggest a potential clinical utility of these compounds. Interestingly, (−) hydroxycitric acid (HCA) which is a known competitive inhibitor of ACLY significantly reduced levels of cholesterol, LDL and triglycerides without apparent side effects in clinical studies. HCA is derived from a subtropical plant, *Garcinia gummi gutta*, which has been consumed as food and traditional medicine in India, suggesting that HCA may be used as chemo preventive food supplement.

3.3. Acetyl CoA carboxylase is associated with tumor progression

ACC is an enzyme of ATP dependent carboxylase and converts acetyl CoA to malonyl CoA which then serves as a substrate for FAS to generate fatty acids. There are two isozymes of ACC (alpha and beta) whose expressions are regulated by a variety of factors such as nutrition, hormones and other physiological responses [131]. The function of ACC alpha appears to be essential for embryonic development as an ACC alpha deficient mouse is embryonic lethal. On the other hand, RNA and protein levels of ACC alpha have been reported to be significantly increased in tumor cells and they were also associated with the up regulation of FAS expression [132]. Interestingly, the amount of phosphorylated ACC (p ACC) was found to be dramatically increased in lung cancer and other "high energy consuming" cells, even though phosphorylated ACC is an inactive form and its expression is related with better survival of cancer patients [26]. It was also reported that inhibition of ACC alpha with a chemical reagent, TOFA (5 (tetradecyloxy) 2 furancarboxylic acid), or shRNA resulted in cell cycle arrest and apoptosis of tumor cells and that this effect was reversed by addition of palmitate in culture medium [27,133]. Palmitate is a structural component of cell membrane and also serves as an energy source; however it also acts as a signaling molecule, although the exact role of this fatty acid in tumorigenesis is yet unclear. Nevertheless, a small molecule which can specifically inhibit the ACC activity is expected to potentially work as an anti cancer drug.

3.4. Fatty acid synthase is up regulated at an early stage of cancer

FAS is a multifunctional enzyme which is composed of seven functional domains (KS, β ketoacyl synthase; MAT, malonyl CoA / acetyl CoA ACP transacylase; DH, dehydratase; ER, β enoyl reductase; KR, β ketoacyl reductase; ACP, acyl carrier protein; and TE, thioesterase) [134 136]. All these activities coordinately synthesize fatty acid using acetyl CoA and malonyl CoA as a primer and a carbon donor, respectively. The FAS gene is abundantly expressed during embryonic development; however, the expression of this gene is restricted to the liver, lactating breast and brain in adult tissues [137 139]. On the other hand, FAS is significantly up regulated in a variety of cancers at an early stage and its expression is positively correlated to poor survival of patients [29 31]. In breast cancer, both FAS and HER2 are expressed at premalignant stage such as DCIS (Ductal Carcinoma *in situ*) [140,141], and their expression tends to be higher in more malignant cells. Importantly, inhibition of FAS expression in tumor cells by siRNA or small chemicals induces cell growth arrest and concomitant apoptosis. Therefore, these results suggest that FAS is involved in the early stage of tumorigenesis, possibly by blocking apoptosis. Indeed, a transgenic mouse which is specifically expressing FAS in prostate has been recently shown to develop *in situ*, non invasive tumor [32]. Although how FAS induces cell transformation is yet to be elucidated, forced expression of FAS was found to stimulate cell growth and reduce sensitivity to tyrosine kinase inhibitors (HER2 inhibitor) *in vitro* [33], suggesting that FAS may exert its oncogenic property by up regulating HER2. Furthermore, it is reported that tumor suppressor protein, PTEN, is capable of suppressing FAS and down regulation of PTEN resulted in significantly higher expression of the FAS gene [29]. Therefore, dysregulation of PTEN which is often observed in breast cancer patients and resultant up regulation of FAS are likely contributing to breast tumorigenesis at an early stage by blocking apoptotic signaling. In this context, it should be noted that inhibition of FAS significantly augmented the expression of pro apoptotic genes including BNIP3, DAPK2 and TRAIL [132].

FAS gene is regulated by several transcriptional factors including sterol regulatory element binding protein (SREBP) which binds to consensus sequence, SRE/E box, on the FAS promoter [142,143]. Interestingly, FBI 1 which is known as a proto oncogene directly interacts with SREBP and synergistically enhances the expression of FAS gene [144], suggesting a link of another oncogene to FAS induced tumor progression. The FAS gene is also regulated by environmental factors. For example, hypoxic condition was shown to cause up regulation of the FAS gene through increase in ROS in cancer cell [143]. Indeed, the over expression of FAS is often observed in hypoxic region of tumors and this up regulation may contribute to tumor progression by

blocking apoptosis signaling and in turn enhancing tumor cell survival as well as promoting chemo resistance.

FAS has been considered an ideal target for cancer treatment due to its specific expression in tumor cells. In fact, treatment of tumor cells with pharmacological inhibitors of FAS such as cerulenin, C75 and Orlistat leads to cell cycle arrest and apoptosis [29,30,34 42]. However, the specificity of action of these inhibitors is still a concern. Cerulenin harbors a highly reactive epoxy group that may also interact with other proteins and may affect processes other than fatty acid synthesis. C75 was designed to be a less reactive (and therefore potentially safer) form of the classical FAS inhibitor, cerulenin [43]. When given i.p., C75 rapidly caused stools to become extremely loose or liquid, and this was accompanied by weight loss, decreased food intake, and inhibition of normal paper shredding behavior of animal. On the other hand, Orlistat appears to be more specific; however, this drug needs to be administered orally and the effects of Orlistat are largely confined to the gastrointestinal tract, where it inactivates pancreatic lipase [44]. This compound is also known as an anti obesity drug and inhibits the thioesterase domain of FAS. Therefore, developing a more specific and less toxic drug to block the function of FAS is necessary and it is currently under intensive study.

One of the unique features of FAS enzyme is its secretory form. A highly sensitive ELISA (FASgen, Inc.) system is indeed available, and it has been reported that the expression level of FAS in serum is strongly associated with tumor stage and survival of patients with a variety of cancers, suggesting the utility of the secreted form of FAS as a diagnostic and prognostic tool [45,46].

4. Mitochondrial enzymes in cancer

Dysregulation of mitochondrial function is a hallmark of cancer cell and several key genes are identified that are closely linked to tumor progression, namely SDH (succinate dehydrogenase), FH (fumarate hydratase) and IDH (isocitrate dehydrogenase). Mutations or loss of SDH and FH genes are known to be oncogenic and they are considered to be tumor suppressors (Table 1, [58 60]). There are four subunits of SDH (A, B, C, D) that are assembled as complex II in mitochondrial electron transport chain. Although complex II gener ally converts succinate to fumarate, mutations of SDHB, SDHC and SDHD cause accumulation of succinate and inhibit PHD (prolyl hydroxylase) function which induces degradation of HIF1α [145]. On the other hand, mutated FH cannot convert fumarate to malate and consequently induces PHD inactivation. Therefore, dysfunction of SDH and FH enzymes cause the activation of HIF1α which enhances tumorigenic related signaling such as angiogenesis. However, the exact molecular mechanism of the tumor suppressive function of SDH and FH is yet to be defined.

The $NADP^+$ dependent IDH gene, which converts isocitrate to α ketoglutarate, is often mutated at amino acid 132 in glioblastomas [146], and the protein level of IDH was found to be frequently increased in many metastatic ductal carcinoma compared to normal cells [147,148], suggesting an oncogenic role for this gene in the mitochondria. IDH is considered as one of the major producers of NADPH which is required for fatty acids and cholesterol biosynthesis; however the transgenic mice of IDH exhibited fatty liver, hyperlip idemia and obesity but not tumor [149]. How IDH contributes to tumor progression is still not clearly defined; however, one attractive theory is that IDH contributes to defense system in cancer cell against ROS which often causes cell death via DNA break. In this context, it should be noted that cancer stem cell has a powerful ROS scavenging system through up regulation of GSH (glutathione) and its related genes such as IDH and Foxo1 which regulates antioxidant related genes [150]. Therefore, IDH may contribute to the maintenance of stemness of tumor stem cell. However this hypothesis needs more rigorous testing.

5. Nucleotide synthesis in cancer

5.1. Nucleotide metabolism in cancer cells

Nucleotides are key components of DNA and RNA structures and they also serve as important sources of cofactors such as CoA and NAD in cellular signaling. Therefore, dysregulation of nucleotide biosyn thesis has profound effects on normal cellular physiology which often result in neoplastic transformation of the cell. When cells become cancerous and highly prolific, they require excess and balanced supply of nucleotides for their growth and survival. However, when this balance is perturbed, DNA gains considerable chances of further mu tations, which leads to more malignant characteristics of the tumor cells. Therefore, nucleotide metabolism plays an important role in tumorigenesis and tumor progression. There are a series of key enzymes that are involved in the nucleotide biosynthesis and modi fication including CTP synthetase, thymidylate synthase, dihydrofo late reductase, IMP dehydrogenase, ribonucleotide reductase, DNA polymerase, and DNA methyltransferase. These enzymes are indeed markedly up regulated in many types of cancer, and therefore, they are considered to be a valid target for cancer therapy [151 158]. Among these enzymes, ribonucleotide reductase and thymidylate syn thase are particularly attractive because the level of these enzymes is highly elevated in various cancers and they are shown to be directly involved in tumor initiation (Table 1, [28,47 57]). Therefore, this section focuses on these two genes and discusses their potential utility for therapeutic targets as well as diagnostic markers.

5.2. Ribonucleotide reductase is double face protein as tumor suppressor and oncoprotein

Ribonucleotide reductase (RNR) which is a key enzyme of rate limiting step in dNDP biosynthesis has been shown to play a critical role in tumorigenesis and tumor progression [159,160]. This enzyme reduces ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs) by tyrosyl radical reaction with Fe(III) cluster. The enzyme is composed of two non identical homo dimeric subunits; RRM1 and RRM2 [47]. The large R1 subunit with a molecular mass of 90 kDa has a catalytic domain and is encoded by the RRM1 gene whose protein level is constant throughout the cell cycle. On the other hand, the small R2 subunit with a molecular mass of 45 kDa has tyrosine residue as a free radical scavenger with diferric iron, which can reduce NDPs to dNDPs. p53R2 (RRM2b) is a homologous gene of the RRM2 with 80% sequence similarity and is originally identified as a target gene of the p53 tumor suppressor protein [161,162]. The expression of RRM2 gene is usually maintained to be higher than that of RRM1 and it reaches a maximum during S phase. The gene is known to be regulated by cell cycle associated transcription factors, such as NF Y and E2F [163,164], and therefore, the cell cycle dependent activity of RNR enzymes is controlled by the level of RRM2. When the expression level of RRM2 is reduced, p53R2 binds to RRM1 subunit to form active RNR complex which can supply dNDPs for repairing damaged DNA. Therefore, RNR has a critical role in DNA repair during cell cycle and their expressions are stringently regulated.

RRM2 and p53R2 are found to be markedly up regulated in many types of cancer cells in patients, indicating the direct roles of these genes in tumor progression [160]. In addition, ectopic expression of the RRM2 gene was shown to increase membrane associated Raf1 expression, MAPK2 and Rac 1 activation, which resulted in enhanced metastatic potential in a xenograft model, suggesting that RRM2 is also involved in tumor progression [48]. In this context, it should be noted that over expression of RRM2 was found to enhance cellular invasiveness through activation of NF kB which increases MMP9 expression [165,166]. To further gain insight into the role of RNR in tumorigenesis, transgenic mice of RRM1, RRM2 and p53R2 have been recently established [47]. The mice over expressing RRM2 and/or

p53R2 in the lungs were found to generate tumors in around 40% of the animals, providing direct evidence to show that these genes indeed act as oncogenes.

On the contrary, RRM1 has a tumor suppressor activity, as shown by gene transfer experiments in both mouse and human cell lines [167]. Ectopic expression of RRM1 in human and mouse lung cancer cell lines significantly up regulated the PTEN gene, suppressed migration and invasion as well as metastasis formation in an animal model [164]. In clinical studies, the median disease free survival exceeded 120 months in the group of patients with tumors that had high expression of RRM1 compared to the patients who had low level of RRM1 [168]. The molecular mechanism of these striking and contrasting differences between RRM1 and RRM2 in their pathogenic roles during tumor progression is not well understood; however, they provide important tools to further investigate the pathological roles of dNDP biosynthesis in tumorigenesis.

Inhibition of nucleotide biosynthesis in tumor cells by anti metabolites is one of the classic approaches for cancer treatments and this approach continues to be effective. RNR inhibitors are classified into several groups as translational, dimerization and catalytic inhibitors. The catalytic inhibitors are further divided into subgroups, inhibitors of sulfhydryl groups, allosteric inhibitors and substrate analogues. Several antisense oligonucleotides (ASOs) specific to RRM2 are in clinical trials. GTI 2040 (combination of capecitabine) has just completed a clinical trial and is already in clinical use for renal cancer. CALAA 01 is a mixture of RNAi and nanoparticle, and therefore, resistant to nuclease deg radation. CALAA 01 is currently in phase I trial (NCT00689065).

5.3. Thymidylate synthase acts as an oncogene by altering nucleotide metabolism

Thymidylate synthase (TYMS) plays a key role in the biosynthesis of thymidine monophosphate (dTMP) which is an essential substrate of DNA synthesis. TYMS is a 74 kDa protein and forms a homodimer which catalyzes reductive methylation of deoxyuridine monopho sphate (dUMP) to generate dTMP using a cofactor, CH_2H_4 folate. Expression of TYMS is controlled by the transcription factor E2F which is linked to cell cycle regulation and proliferation [169,170], and inhibition of this enzyme results in cell arrest. The results of microarray and immunohistochemical studies indicate that the expression of this enzyme is significantly up regulated in various tumors including breast, bladder, cervical, kidney, lung and gastrointestinal cancers [171 176]. The high expression of TYMS is also associated with poor clinical outcomes in these cancers, suggesting that TYMS acts as an oncogene. In fact, ectopic expression of TYMS has been shown to confer normal cell with transformed and tumorigenic phenotype in a xenograft model [54]. Notably, the elevated level of TYMS expression was also shown to result in more invasive and metastatic abilities in these cells. Furthermore, a recent study of TYMS transgenic mice revealed that over expression of this gene caused pancreatic islet hyperplasia and islet cell tumors [55]. Importantly, mutations at the active site of this enzyme diminished the ability of tumor formation in mice, suggesting that an imbalance of nucleotide pools by increasing levels of TYMS enhances mutations and thereby causes oncogenic transformation.

TYMS has been recognized as an effective target for anti cancer therapy, and several inhibitors of TYMS have been used clinically for over 30 years. Among these drugs, 5 fluorouracil (5 FU) has been widely used for many types of cancer; however, 5 FU is known to have unwanted side effects due to its broad specificity. Recently, several analogues of folates have been developed as a new class of TYMS inhibitors, and some of them are currently in clinical trials. Raltitrexed (RTX, Tomudex or ZD1694) is in various phases of clinical trials with combination of several anti cancer drugs for solid tumors, colon and rectal cancers and leukemia, and another antifolate drug, ZD9331, has completed phase II trial for the treatment of ovarian cancer (NCT00014690). Although these drugs appear to be effective, their potential long term side effects are of some concern because they generally have broad specificity, and developing more specific small chemicals is needed to generate more effective therapeutic drugs.

6. Diagnostic value of metabolic genes

Due to the high level of expression at various stages of tumor tissues, the metabolic genes are considered to serve as diagnostic as well as prognostic markers to predict patient outcome. Immunohis tochemistry is still the most reliable method to examine this possibility, but it is generally not quantitative or cost effective for testing a "signature" of multiple genes at clinical setting. However, recent availability of database of microarray analyses allows us to easily assess the diagnostic value of any combination of genes or "signature" for various types of cancer. Fig. 2 shows such analyses for four different types of tumors including breast, prostate, lung and colon cancer, using GEO microarray database, and the analyses include at least five different database for each cancer type. In breast cancer, PGPI, ACLY, RRM2 and TYMS genes are highly up regulated in at least four different cohorts out of seven independent studies. In prostate cancer, FASN and RRM2 were markedly up regulated in all database, while TKTL1, PGI, ACLY and TYMS are also significantly expressed in at least 3 cohorts out of 5 studies. In lung cancer, four genes including GLUT1, ACLY, RRM2 and TYMS were significantly up regulated in at least 5 independent studies. Moreover three genes including PGI, ACLY and RRM2 genes were up regulated in colon cancer of all studies. These data indicate that a different combination of metabolic genes may serve as "signature" for each type of cancer. We then examined whether a signature of the metabolic genes could predict disease free survival in four independent studies of breast cancer. Fig. 3 indeed indicates that the signatures of PGI, ACLY, RRM2 and TYMS genes have a strong predictable value for breast cancer patient outcome, which is consistent with the notion that these metabolic genes act as oncogenes in breast cancer. Therefore, it is expected that further analysis of different combinations of metabolic genes may reveal a "signature" with more predictable values for each type of cancer.

7. Conclusions and perspectives

During the course of tumor initiation and progression, cancer cells need to reprogram their metabolic pathways in order to respond to the demanding requirements for their own growth. This re program ming is accomplished by both genetic and epigenetic alterations of various metabolic genes, and the dysregulations of some of these genes are directly involved in the initial step of transformation while others contribute to maintenance and acceleration of malignant phenotypes. However it is still not clear how and when these changes occur in normal cells. For example, the dysregulation of the FAS gene is often observed at very early stages of cancer and in benign tumors, suggesting the direct role of this gene in tumor initiation. However, how and what causes this dysregulation remains unknown, and understanding the mechanism and identifying the factors contribut ing to these changes is of paramount interest. It is suspected that not only carcinogens, but also dietary factors, hormonal balance, inflammatory conditions and tumor microenvironment such as stroma and ECM are all likely to be involved in the re programming process of metabolic pathways. In this context, it is worth noting that some metabolic abnormalities such as diabetes and even ageing are linked with higher incidence of cancers. However, whether these abnormalities are directly involved in tumorigenesis remains to be determined.

It is well established that HIF1, AMPK and LKB play central roles in keeping the balance of cell metabolism for survival and growth under

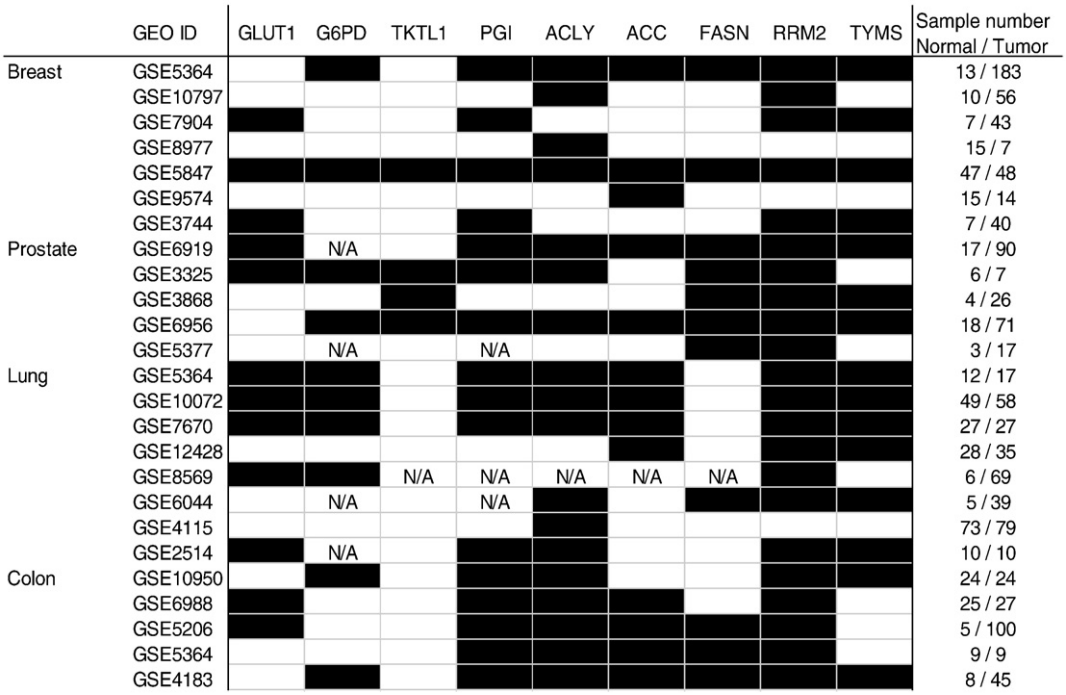

Fig. 2. The expression profile of metabolic genes in clinical samples. The expressions of nine metabolic genes in four different types of cancers (breast, prostate, lung and colon) were examined using the GEO microarray database, and identity of each cohort was shown by GEO ID number. A closed box indicates significantly positive expression ($p<0.05$), and the number of normal and tumor samples in each cohort was also shown in the right column. N/A; not available.

various stressful environments such as hypoxic, acidic and low nu trient conditions. Furthermore, recent findings indicate that the sen sitivity of tumor cells to dietary restriction is closely associated with activation of PI3K pathway, suggesting a key role of this pathway in balancing metabolic homeostasis. It is also noted that the PI3K/AKT pathway directly controls lipogenesis by up regulating SREBP1 which is considered to be a master control gene of various lipogenic genes [177]. Oncogenes that encode transcription factors are also actively involved in regulation of cellular metabolism. For example, Myc is known to regulate Glut1 and other genes in glycolysis. Myc can directly bind the promoters of these genes with other cofactors (e.g. E2F1 for nucleotide metabolism and HIF1 for glucose metabolism) and significantly up regulate the target genes. Therefore, metabolic changes in tumor cell are also modulated by activation of these oncogenes; however, dissecting the exact molecular mechanism of this process is critically important in order to identify a specific target for both preventive and therapeutic intervention.

Another key question is homeostasis and crosstalk of each path way after the re programming in cancer cells. For example, dysregu lation of the Glut1 gene affects not only the glycolytic pathway but

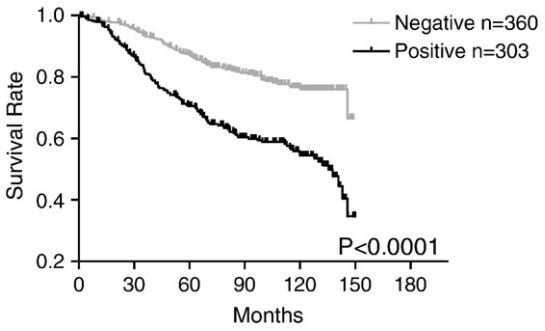

Fig. 3. Kaplan–Meier analysis of metabolic genes for breast cancer patients. Microarray data of 663 breast cancer patients from four independent cohorts were normalized and examined by meta-analysis for PGI, ACLY, RRM2 and TYMS. p value was calculated by log-rank test.

also lipogenesis because glycolysis generates substrate for TCA cycle which ultimately provides a precursor for lipogenesis. In addition, glycolysis and lipogenesis are closely linked through the redox path way. Despite ill functioning mitochondria and the Warburg effect, the balance of these pathways is still well maintained in the cancer cells during their survival and aggressive growth, suggesting that an alternative balancing mechanism needs to be in place, although how this compensatory mechanism works is yet to be defined.

The higher glucose uptake is indeed observed in the majority of tumor cells as originally found by Otto Warburg. However, impair ment of mitochondrial function in cancer cells is still a controversial issue. These conflicting observations are perhaps due to the different experimental approaches including different tumor cell lines, culture methods (mostly monolayers) and assay procedures of mitochondrial functions. Rodríguez Enríquez et al. recently addressed this question using spheroid tumor model [178]. The authors showed that mito chondrial activities markedly decreased in a late stage of spheroid, whereas an activity of glycolysis significantly increased with con comitant over expression of HIF1. Therefore, the level of mitochon drial function appears to be dependent on the stage of tumor growth and location of each cell in tumor mass. The central region of tumor mass is often necrotic and hence under hypoxic condition which profoundly affects the balance between mitochondrial function and glycolytic flux. It is also known that stabilization of HIF1α is induced by oncogenes such as Ras, Src and Myc followed by stimulation of aerobic glycolysis. In addition, the expression level of H+ ATP synthase, a key enzyme of oxidative phosphorylation, was shown to be significantly decreased in lung cancer cells, and blocking the enzymatic activity promoted glycolytic flux [179]. Furthermore, the inhibition of the same enzyme enhanced cell survival by attenuating ROS which is known to control apoptosis [180], while augmentation of mitochondrial metabolism induced suppression of tumor growth [181]. Fantin et al. also showed that enhancement of mitochondrial metabolism by inhibiting LDHA diminished tumorigenicity of cancer cells [182]. These results suggest that mitochondrial function is likely to be intact in tumor cells; however, the level of their activities is heavily dependent on their microenvironment, mainly on availability

of oxygen in the tumor mass, and that different tumor cells adapt different survival strategies and energy metabolism even in the same tumor mass by changing the balance between glycolysis and oxidative phosphorylation.

Re programming of metabolic pathways in cancer stem cell is another important aspect of recent tumor biology and has critical therapeutic implication. Are the metabolic genes already mutated in cancer stem cells? If so, when and how does it occur? How do they affect the abilities of self renewal and differentiation of the cancer stem cell? What are the roles of niche or microenvironment in the metabolism of the tumor stem cell at both primary and metastatic sites? Answers to these questions are virtually unknown at present; however, understanding the underlining mechanism of metabolic re programming in cancer stem cell may provide important clues for novel therapeutic targets. It should be noted that cancer stem cell is likely to be responsible for chemo resistance according to the recent stem cell theory. Therefore, elucidating these questions may also provide us with a tool to overcome the problem of chemoresistant cancers.

Identifying small chemicals to specifically intervene in metabolic pathways and inhibit the function of metabolic genes is considered to be a promising approach to develop a novel type of anti cancer drug, and it is under active investigation. Some of these compounds, such as 2DG for glycolysis and CALAA 01 for nucleotide metabolism, are already in clinical trials. For lipogenesis, FAS is particularly an at tractive target because of its specific expression in various types of cancers and blocking this enzymatic function is known to induce tumor cell apoptosis. However, considering the balancing and com pensatory mechanism of each pathway in cancer cells, simultaneous blocking of multiple pathways instead of targeting a single gene is likely to be a more effective approach. Because the metabolic genes are mostly up regulated at an early stage of cancer, they are also considered to be ideal targets for chemo prevention which is by far the most cost effective way to fight cancer. Some of the metabolic genes and their products are also likely to serve as useful diagnostic tools, and FAS and PGI are promising examples. Perhaps more ag gressive proteomics approach using serum and urine from a cohort of patients with various cancers may identify better diagnostic markers of metabolic pathways.

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Review

Drug development against metastasis-related genes and their pathways: A rationale for cancer therapy

Megumi Iiizumi [1], Wen Liu [1], Sudha K. Pai, Eiji Furuta, Kounosuke Watabe *

Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University, School of Medicine, 801 N. Rutledge Street, P.O. Box 19626, Springfield, Illinois, 62794-9626, USA

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ABSTRACT

It is well recognized that the majority of cancer related deaths is caused by metastatic diseases. Therefore, there is an urgent need for the development of therapeutic intervention specifically targeted to the metastatic process. In the last decade, significant progress has been made in this research field, and many new concepts have emerged that shed light on the molecular mechanism of metastasis cascade which is often portrayed as a succession of six distinct steps; localized invasion, intravasation, translocation, extravasation, micrometastasis and colonization. Successful metastasis is dependent on the balance and complex interplay of both the metastasis promoters and suppressors in each step. Therefore, the basic strategy of our interventions is aimed at either blocking the promoters or potentiating the suppressors in this disease process. Toward this goal, various kinds of antibodies and small molecules have been designed. These include agents that block the ligand recepter interaction of metastasis promoters (HGF/c Met), antagonize the metastasis promoting enzymes (AMF, uPA and MMP) and inhibit the transcriptional activity of metastasis promoter (β Catenin). On the other hand, the intriguing roles of metastasis suppressors and their signal pathways have been extensively studied and various attempts have been made to potentiate these factors. Small molecules have been developed to restore the expression or mimic the function of metastasis suppressor genes such as NM23, E cadherin, Kiss 1, MKK4 and NDRG1, and some of them are under clinical trials. This review summarizes our current understanding of the molecular pathway of tumor metastasis and discusses strategies and recent development of anti metastatic drugs.

Contents

* Corresponding author. Tel.: +217 545 3969; fax: +217 545 3227.
E-mail address: kwatabe@siumed.edu (K. Watabe).
[1] MI and WL contributed equally to this article.

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1. Introduction

Cancer is the second leading cause of death in the USA, and more than half a million people succumb to the disease every year [1]. Despite significant improvements in screening methods and treat ment options, the majority of cancer patients are still diagnosed at an advanced stage, and more than 90% of patients ultimately die from sequel of metastatic disease. Therefore, metastasis is a hallmark of malignancy, and no effective therapeutic option is currently available for those patients. Although the clinical importance of tumor metastasis is well recognized, advances in understanding the molecular mechanism involved in metastasis formation have lagged behind other developments in the field of cancer research. This is attributed to the fact that cancer cells are extremely heterologous in nature and that metastasis involves multiple steps with a high degree of complexity, and each step requires coordinated action of many promoters and suppressors. However, extensive efforts in the past decade have led to the discoveries of many previously unknown factors involved in metastasis and also unveiled several novel concepts in this research field [2,3]. These findings have shed new light on molecular pathways of metastasis, which also provided valuable information about potential targets for the treatment of metastatic disease. This review discusses our current understanding of molecular mechanism of metastatic process and summarizes recent information of drug development specifically targeted to the meta static pathways.

2. Tumor metastasis involves multi-step process with high complexity

A primary tumor generally consists of heterogeneous cell types including a small number of cancer stem cells that are able to perpetually proliferate without responding to tumor suppressor function. The current theory predicts that these cancer stem cells originate from a normal stem cell or a cancer cell, which acquired a stem cell like ability [4]. When a tumor grows more than 1 mm^3 in size at the primary site, it acquires active supply of oxygen and nutrients by promoting angiogenesis. Tumor cells accomplish this task by generating hypoxic environment followed by secretion of angio genic growth factors (Fig. 1). Tumor cells that gain growth advantage further proliferate and acquire metastatic phenotypes due to addi tional mutations. The first step in metastasis is the detachment of these tumor cells from the primary tumor mass by acquiring an invasive phenotype that results in the loss of cell cell adhesion and cell extracellular matrix adhesion followed by proteolytic degradation of the matrix (Fig. 1) [5]. It is believed that autocrine motility factor (AMF) and hepatocyte growth factor (HGF) are critical components of motility and that degradative enzymes including serine , thiol proteinases, heparanases and metalloproteinases such as MMP2 and 9 play critical roles in the invasion [6 8]. When tumor cells intravasate surrounding tumor vasculature and neighboring lymphatic vessels, they must survive in this hostile environment that includes mechan ical damage, lack of growth factor from the original environment and the host immune system (Fig. 1) [9]. Tumor cells in the circulation often aggregate with platelets and fibrin, and they embolize in the capillaries or directly adhere to the endothelial cells by a mechanism similar to leukocyte adhesion at the inflammatory site [10 12]. In some cases, arrested tumor cells extravasate before proliferating themselves using the same hydrolytic enzymes that are used in the initial step of invasion (Fig. 1) [13]. However, in many cases, cancer cells actually proliferate within the lumen of vessels to create a considerable tumor mass that can eventually obliterate the adjacent vessel wall by pushing aside the barrier composed of endothelial cells, pericytes and smooth muscle cells that previously separated the vessel lumen from the surrounding tissue [14,15]. After extravasation, cancer cells lodge at the secondary sites, where the cells must also proliferate and colonize for successful metastasis (Fig. 1). These processes are controlled by various metastasis promoters and suppressors, and they must be well coordinated to establish successful distant metastasis (Table 1) [2]. Recent advancement of research in this field has revealed the complex interplay of metastatic factors and many novel concepts of signal pathways leading to metastasis (Fig. 2 a,b). Based on this information, the current research is gradually moving toward translational stage by aiming at development of targeted anti metastatic drugs (Table 1). The following sections summarize up to date information of the promoters and suppressors of metastasis that are currently under active investigation for drug development.

3. Metastasis promoters

3.1. Amf

Autocrine motility factor (AMF) was originally isolated as a C *X X* C cytokine that stimulates random or directed motility of AMF

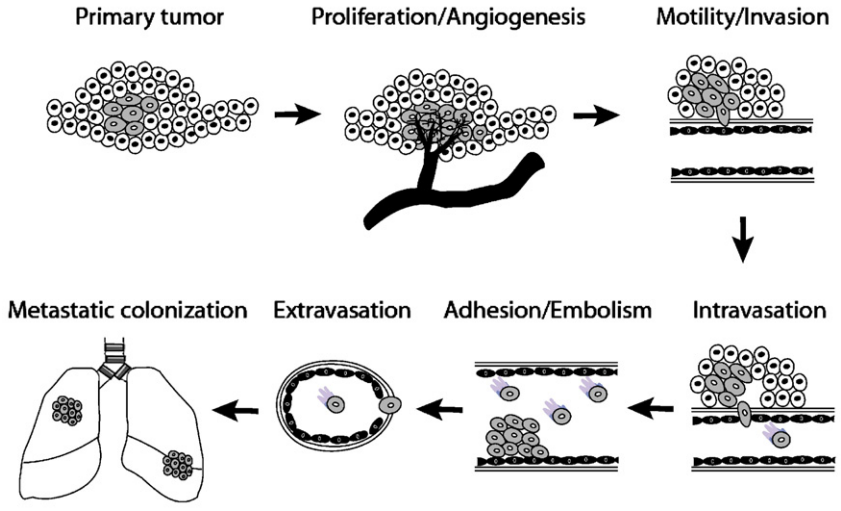

Fig. 1. Process of tumor metastasis. As primary tumor grows, tumor cells induce angiogenic factors to promote vessel formation which facilitates tumor growth and cell invasion into the circulatory system. Some tumor cells gain an invasive ability by expressing motility factors and proteases followed by breaching the basement membrane. Tumor cells then enter the blood vessel where they often aggregate with the platelets and cause embolize. When cells migrate to a distant organ, they adhere to endothelial cells and extravasate by inducing proteases. Cells then colonize and establish metastasis at the distant organ site where appropriate growth factors are provided.

producing tumor cells in an autocrine manner [16]. Elevated serum AMF was found in patients with malignant tumors such as colorectal, lung, kidney, breast and gastrointestinal carcinomas and is well correlated with the development of metastasis [16 19]. AMF is a multifunctional molecule, also known as phosphoglucose isomerase, neuroleukin, and maturation factor [20]. AMF causes tumor cell detachment from the primary site by promoting cell motility in an autocrine fashion. However, recent research revealed that AMF also contributes to malignant progression by stimulating the migration and proliferation of endothelial cells via its receptor AMFR, a unique seven transmembrane receptor (gp78), followed by activation of small Rho like GTPase [16,21]. Therefore, tumor cells appear to induce aggressive angiogenesis by promoting cross talk of signals between VEGF VEGFR and AMF AMFR which also promotes cell survival via activation of Akt and MAPK dependent anti apoptotic pathways (Fig. 2) [22]. A recent report by Raz et al. demonstrated a more direct role of AMF in tumor progression and metastasis. They have shown that over expression of AMF in normal fibroblasts lead to a gain of tumor igenicity, whereas down regulation of AMF by siRNA in mesenchymal tumor cells resulted in mesenchymal to epithelial transition (MET), the reverse process of epithelial to mesenchymal transition, as reflected by a loss of cell polarity, reduced proliferation and invasion *in vitro* and loss of tumorigenic properties *in vivo* [23]. Interestingly, they later also showed that silencing AMF expression in human fibrosarcoma cells resulted in an increased sensitivity to oxidative stress induced and p21 mediated cellular senescence, which brought a novel insight into the function of AMF in tumor progression [24]. Collectively, neutralizing AMF, disruption of AMFR and blocking their signal pathways are considered to be rational approaches for anti metastatic drug development.

It has been shown that specific carbohydrate phosphate inhibi tors including E4P, D mannose 6 phosphate and 5 phospho D arabinonate (5PAA) are able to block both AMF enzymatic activity and AMF induced cell motility [25,26]. Treatment of tumor cells with these inhibitors has been shown to decrease the growth, DNA synthesis, migration and invasiveness of several types of cancer cells [22,23,27]. Since these carbohydrate phosphate inhibitors are among the smallest compounds that have AMF inhibitory activity, informa tion of the known crystal structure may help in designing a lead compound to develop more effective AMF inhibitors.

Because AMF is a secretory factor, antibody against AMF may also be a rational approach. In fact, Talukder et al. showed that neutralizing antibodies against AMF were able to partially block HRG induced invasiveness of human breast cancer MCF 7 cells [28]. Raz et al. also demonstrated that a monoclonal anti AMF antibody induced apopto sis in human fibrosarcoma cell lines *in vitro* and effectively promoted drug induced apoptosis *in vivo* [22]. Therefore, humanized anti AMF holds promise for future therapeutic application. Interestingly, anti body against EGFR2 (Herceptin) was also shown by Talukder et al. to block AMF expression and its promoter activity [27]. Because Herceptin has been used as an effective drug for breast cancer, it is interesting to know whether this antibody also blocks the invasive ness of the tumor.

Ectopic expression of AMF makes some tumor cells become resistant to apoptosis induced by serum deprivation, and this resistance appears to be mediated via PI3K and PKC/MAPK pathways (Fig. 2A). Yanagawa et al. recently indeed showed that PI3K inhibitors (Ly294002 and Wortmanin), PKC inhibitor (GF109203) and MAPK inhibitor (PD98059) were able to recover the expression of Apaf 1 (Apoptotic protease activating factor 1) in the AMF transfected HT1080 cells followed by induction of apoptosis [22]. In addition, GF109203X and Wortmanin were shown to inhibit AMF induced expression of fms like tyrosine kinase (Flt 1) and hence impair the proliferative signals of VEGF in endothelial cells. Therefore, AMF may be a good target for anti angiogenic therapy, although potential side effects of such drugs are unknown. Finally, it is recently found that the stability of AMF protein is regulated through ubiquitin lysosome system, which is mediated by poly (ADP ribose) polymerase 14 (PARP 14). This new discovery may offer a novel target to block the AMF/AMFR signaling and deserves further investigation [29].

3.2. Hgf/sf

Hepatocyte growth factor (HGF), also known as scatter factor (SF), was identified as the natural ligand for the c Met receptor tyrosine kinase [30]. HGF/SF interacts with c Met receptor and transduces multiple biological signalings that control proliferation, disruption of intercellular junctions of EMC, migration and protection from apoptosis [31,32]. HGF/SF signaling has also been demonstrated to play an important role in a wide variety of human cancers of both epithelial and mesenchymal origins [31]. The results of several clinical studies indicate the prognostic value of HGF/SF and c Met in various types of cancer and that the expression of HGF and/or c Met is frequently associated with the aggressive nature of the tumors and the poor clinical outcome [31,33]. The exact mechanism of up regulation of these genes in cancer is not well understood. However, a recent study suggested that the up regulation of c Met and HGF may be due to the stress of tumor microenvironment such as hypoxia [34]. Therefore, HGF/SF is considered to be widely involved in the tumor metastatic process. HGF is a potential promoter of cell invasion by directly stimulating the motility and migration of cancer cells as well as affecting the microenvironment [32]. HGF can disrupt cell cell adhesion and promote cancer cell growth, partly by inducing phosphorylation of β Catenin and relocation of E cadherin, which may result in down regulation of cell cycle regulatory factors such as p27 (Fig. 2A) [35 37]. On the other hand, HGF can increase the adhesion between cancer cells and matrix by activating the FAK and paxillin pathways, which cooperatively regulate the expression of integrins in cancer cells and eventually lead to adhesion as well as migration of cancer cells to matrix [38]. HGF is also able to increase the expression and secretion of proteolytic enzymes from cancer cells including MMP2, MMP7, MMP9 and uPA that are involved in matrix and basement membrane degradation (Fig. 2) [36,39,40]. In addition, HGF is considered as an angiogenesis promoting factor through its direct morphogenic and adhesive effects and indirect regulation of other angiogenic factors such as IL 8, VEGF and TSP 1 [41,42]. Furthermore, Boccaccio et al. have recently demonstrated that the c Met oncogene was responsible for the induction of thrombohemor rhagic syndrome, suggesting that c Met may give survival advantage to tumor cells in the circulation by promoting the aggregation of tumor cells with platelets [43,44]. Therefore, the HGF/c Met signaling plays a critical role in the metastatic process and this gene as well as the downstream signal can be potential targets for cancer therapy.

Recently, rapid progress has been made toward drug development against HGF/SF for the purpose of cancer therapy. These include HGF antagonists, anti HGF and anti cMet antibodies, small molecules targeting c Met and its signaling pathways as well as compounds interfering with HGF elicited biological activities [45]. Antagonizing ligand binding that block the activation of downstream signaling is a conventional therapeutic strategy for most carcinomas. NK4 is one of the antagonists that compete with HGF for the c Met receptor, and it has been known to block HGF induced cellular adhesion, invasion and metastasis in various types of cancer cells including breast, bladder, colorectal, lung, prostate, glioma, pancreatic and gastric cancers *in vitro* [46]. Moreover, NK4 also acts as angiogenesis inhibitor, and this activity is independent of its action as HGF antagonist [47,48]. As expected, treatment of mice via intraperitoneal or intratumoral administration of NK4 protein or recombinant adenoviruses expres sion vector effectively blocked tumorigenesis, angiogenesis and metastasis in various mouse xenograft models including pancreatic and gastric cancers [46,49]. Another antagonist is an uncleavable HGF, which was engineered with a single amino acid substitution at the

Table 1

Metastasis promoter	Drug	Original target	Action	Animal	Clinic trial	Reference
AMF	carbohydrate phosphate compounds (E4P,M6P,5PA)	AMF	Inhibit AMF cytokine enzymatic activity		Pre-clinical studies	[25,26]
	Herceptin	EGFR2	Down-regulates AMF protein and promoter activity	Increase the tumor progression time in mice model of xenograft tumor of Her2 over-expression	In clinical use	[27,270]
HGF/c-Met	NK4	HGF	competitive antagonist for HGF binding to the c-Met receptor	Inhibited tumorigenesis, angiogenesis and metastases in mouse tumor xenograft models	Pre-clinical	[46,49]
	uncleavable HGF	HGF	Prevent maturation of pro-HGF and compete with HGF to bind to c-Met receptor	Inhibited tumor growth, angiogenesis and metastases in tumor xenograft models	Pre-clinical	[50]
	AMG102	HGF	Neutralizing anti-HGF antibody	pharmacokinetic and safety profile are passed through in cynomolgus monkeys test	Phase II	[53]
	DN30	c-Met	Binds to extracellular domain of c-Met and prevent its activation	inhibited growth and metastatic spread to the lung of tumor xenograft mouse model	Pre-clinical	[61]
	PHA-665752 SU11274 K252a	Kinase inhibitors	inhibit c-Met phosphorylation	Inhibition of tumor growth in c-Met-dependent lung and gastric carcinoma xenograft animal model	Pre-clinical	[55-60]
TGF-β	SD-208	TGFβ1 receptor	TGF-β typeI receptor kinase inhibitor	Inhibited primary tumor growth, angiogenesis and metastasis of xenograft animal model	Pre-clinical studies	[68,73,87-92]
	SD-093 SB-431542 A-83-01 LY2109761					
	2G7	TGFβ	Neutralizing antibody of TGFβ	Inhibited abdominal and lung metastasis of xenograft animal model	Pre-clinical	[69]
	β- glycan (sRIII)	TGFβ	Soluble extracellular domain of TGF-β type III receptor	Inhibited lung metastasis in human breast tumor xenograft model	Pre-clinical	[96]
	Fc:TβRII	TGFβ	Dominant negative TGF-β typeII receptor	Inhibited lung metastasis in human melanoma xenograft model and MMTV-Neu model	Pre-clinical	[94,95]
	AP12009	TGFβ	Oligonucleotide against human TGFβ2		PhaseI/II (high grade glioma)	[97]
MMP	Marimastat (BB-2516)	MMPs	Pharmacologically developed MMPs inhibitor		PhaseII,III,IV (Pancreatic cancer) phaseIII Non-small-cell lung cancer)	[122,271]
	Prinomastat (AG3340)	MMPs	inhibitor with selectivity for MMPs 2, 3, 9, 13, and 14	enhance tumoricidal activity after Photodynamic therapy in a mouse mammary tumor model	Phase III, IV (NSCLC) phaseII (advanced esophageal cancer)	[122,272]
	Tanomastat(BAY12-9566)	MMPs	Pharmacologically developed MMPs inhibitor		PhaseIII (Small-cell lung and pancreatic cancer)	[122,271]
	BMS-275291Neovastat	MMPs	Pharmacologically developed MMPs inhibitor		PhaseIII, IV (Non-small-cell lung and Renal cell carcinoma)	[122,271]
	Bisphosphonates (BP)	for use in disorders of bone metabolism	Inhibit proteolytic activity of MMPs	Increase bone mineral density in animal model	In use (osteolytic metastases)	[129]
uPA	WX-UK1 WX-671	uPA	Protease inhibitor		Phase I,II	[148]
	231 Bi-PAI2	uPA	Recombinant PAI-2 (uPA inhibitor-2)	Inhibited micrometastasis in human breast cancer xenograft models	Pre-clinical studies	[160-162]
	1-Isoquinolinylguanidines (UK-356,202) and its derivatives	uPA	Reversibly competitive inhibitors of uPA enzymatic activity	Inhibit exogenous uPA in human chronic wound fluid and in the porcine excisional wound model	Pre-clinical studies	[273]
	Bikunin	Trypsin and plasmin	Down-regulate uPA gene and protein expression	once-daily oral administration of bikunin against ovarian carcinoma in nude mice	Phase I	[153-156]
	DX-1000 PEGylated DX-100	plasmin	Down-regulate uPA expression	Inhibited tumor proliferation and vascularization in human tumor xenograft model	Pre-clinical	[157,158]
β-catenin	Celecoxib	COX-2	Induce degradation of β-catenin via a COX-2-independent mechanism	Diet treatment significantly reduce tumor development without signs of metastasis in TRAMP mice	phase II (advanced colorectal cancer)	[182,183,274]
	R-Etodolac and its analog (SDX-308)	enantiomer of Etodolac	Down-regulates protein and promoter activity, increase β-catenin and E-cadherin complex at the membrane	inhibited tumor development and metastasis in the transgenic mouse adenocarcinoma of the prostate (TRAMP) model	phase II (chronic lymphocytic leukemia)	[182,183]
	Thiazolidinedione (TZD)	PPARs	cause localization shift to cytoplasm, reduced tyrosine phosphorylation of beta-catenin	Inhibited lymph node and lung metastases in the xenograft animal model	Pre-clinical studies	[185]

Table 1 (*continued*)

Metastasis promoter	Drug	Original target	Action	Animal	Clinic trial	Reference
β-catenin	Exisulind(Aptosyn)	SAANDs	Down-regulate β-catenin and cyclin D1 via PKG-mediated signalling	Inhibited tumor growth and metastasis of human lung cancer xenograft in athymic nude rats.	Phasae I,II,III	[164,172,173]
	CP461 CP248					
	Imatinib (Gleevec)	PDGF receptor	Inhibits tyrosine phosphorylation of β-catenin and resultant cell migration		In use (chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) etc)	[176]
Metastasis Suppressor NM23	Medroxyprogesterone acetate (MPA)	Progesterone receptor	MPA elevated NM23 expression and inhibited soft agar colonization	Inhibited lung cancer metastasis in the experimentally metastasis mice model	Phase III(metastatic breast cancer)	[206,275,276]
	Estradiol	Estrogen receptor	Up-regulates NM23-H1 in ERa+ breast cancer cell lines. Inhibits invasion *in vitro*.	Suppression of lung metastasis *in vivo* model of chemically induced hepatocellular carcinoma.	Phase II (metastatic breast and prostate cancer)	[212,277]
	Aspirin	Cox1/2 inhibitor	Up-regulates NM23. Decreased metastatic phenotype *in vitro*.		Phase III (esophageal cancer)	[217,277]
	Indomethacin	Cox1/2 inhibitor	Up-regulates NM23 expression in breast cancer cell lines	Inhibited lung tumor metastasis in the experimental metastasis mice model	Phase II (head and neck cancer)	[219,220,277]
	All-trans retinoic acid (ATRA)	Retinoid receptors	Up-regulates NM23 in hepatocarcinoma cells. Increased adhesion to ECM *in vitro*	Inhibits the growth of xenograft tumors and gastric cancer cell metastasis to liver.	Currently in clinical use, (acute promyelocytic leukemia)	[226-228,278]
KiSS-1	Metastin	orphan G-protein coupled receptor	Regulate the NFκB signaling pathway		Pre-clinical studies	[279]
MKK4	Anti-death receptor antibody (2E12, TRA-8)	death receptor	Induce apoptosis *in vitro*. Activate MKK4/JNK/p38 pathways		Pre-clinical studies	[241]
	Bisindolylmaleimide VIII	PKC inhibitor	Enhances affects of anti-death receptor antibodies		Pre-clinical studies	[245]
E-cadherin	pyrazolo [3,4-d] pyrimidines (PP)1, PP2	Src family inhibitor	Reactivate the E-cadherin expression. Reduced migration ability of breast cancer cells	Decrease in pancreatic tumor growth and metastasis in nude mice	Pre-clinical studies	[255,256,280]
NDRG1	Fe chelator (DFO, 311)	Fe	NDRG1 was specifically up-regulated by Fe chelation.	Delay or regression of tumor cell growth in athymic nude mice.	Phase II (Neuroblastoma)	[263,266,281,282]

proteolytic site of HGF [50]. The uncleavable HGF competes with endogenous pro HGF for the catalytic domain and thus inhibits endogenous pro HGF maturation. The peptide also binds to the c Met receptor with high affinity and displaces the mature ligand. More strikingly, both local and systemic administration of uncleavable HGF in a xenograft mouse model significantly suppressed tumor growth and tumor angiogenesis, and notably inhibited the formation of spontaneous metastases without affecting vital physiological func tions [50]. In a separate study, neutralizing anti HGF antibodies were first developed by Cao et al. who demonstrated that a minimum of three antibodies, each of which act on different HGF epitopes, were required to block c Met tyrosine kinase activation and the biological outcomes [51]. Moreover, Burgess et al. have shown that fully humanized monoclonal anti HGF antibodies effectively suppressed HGF dependent tumor growth in tumor xenograft mouse model [52]. Another fully human HGF antibody, AMG102, was recently tested for its pharmacokinetics and safety in monkeys and further clinical investigation was warranted [53].

It is recently suggested that MET functions in certain human cancers as “oncogene addiction”, the concept formulated in the late 1990s, indicating a constant requirement of MET in these tumors [54]. Therefore, targeting the activated c Met holds a great promise as an anti cancer therapy at least for certain tumor types. Regarding c Met tyrosine kinase receptor inhibitors, a set of low molecular weight compounds including PHA 665752, SU11274, and K252a, which are able to compete for the ATP binding and prevent receptor transactiva tion and recruitment of the downstream effectors, have recently been tested and shown to effectively inhibit the kinase activity and block the subsequent signaling pathways [55 58] . Particularly, PHA 665742 is capable of inhibiting the autophosphorylation of c Met with a relatively high specificity compared to other tyrosine and serine threonine kinases [55,59]. In addition, PHA 665752 was shown to induce massive apoptosis in human gastric cancer cell lines that had amplified MET genes, while it did not affect other cell lines without c Met receptor amplification [59]. Furthermore, Salgia et al. has recently shown that PHA 665752 treatment inhibited tumorigenicity and angiogenesis in a mouse model of lung cancer xenografts [60]. These results strongly support a potential utility of these compounds for a therapeutic application in the future. Designing a drug that binds the extracellular domain of the c Met receptor and thus impairing receptor dimerization has been considered as another c Met blocking strategy. Recently, Petrelli et al. showed that a monoclonal antibody, DN30, prevented c Met activation and abrogated its biological activity [61]. In addition, soluble recombinant Sema proteins or anti Sema antibodies against the extracellular Sema domain that is involved in ligand binding and receptor dimerization of c Met have been generated [62]. As expected, they suppressed the downstream signaling triggered by the c Met receptor even in the presence of HGF. Another alternative strategy for specifically blocking the receptor is a gene silencing technology. Using adenovirus vectors carrying small interfering RNA targeting c MET, Shinomiya et al. demonstrated that the siRNA drastically reduced the c MET gene expression

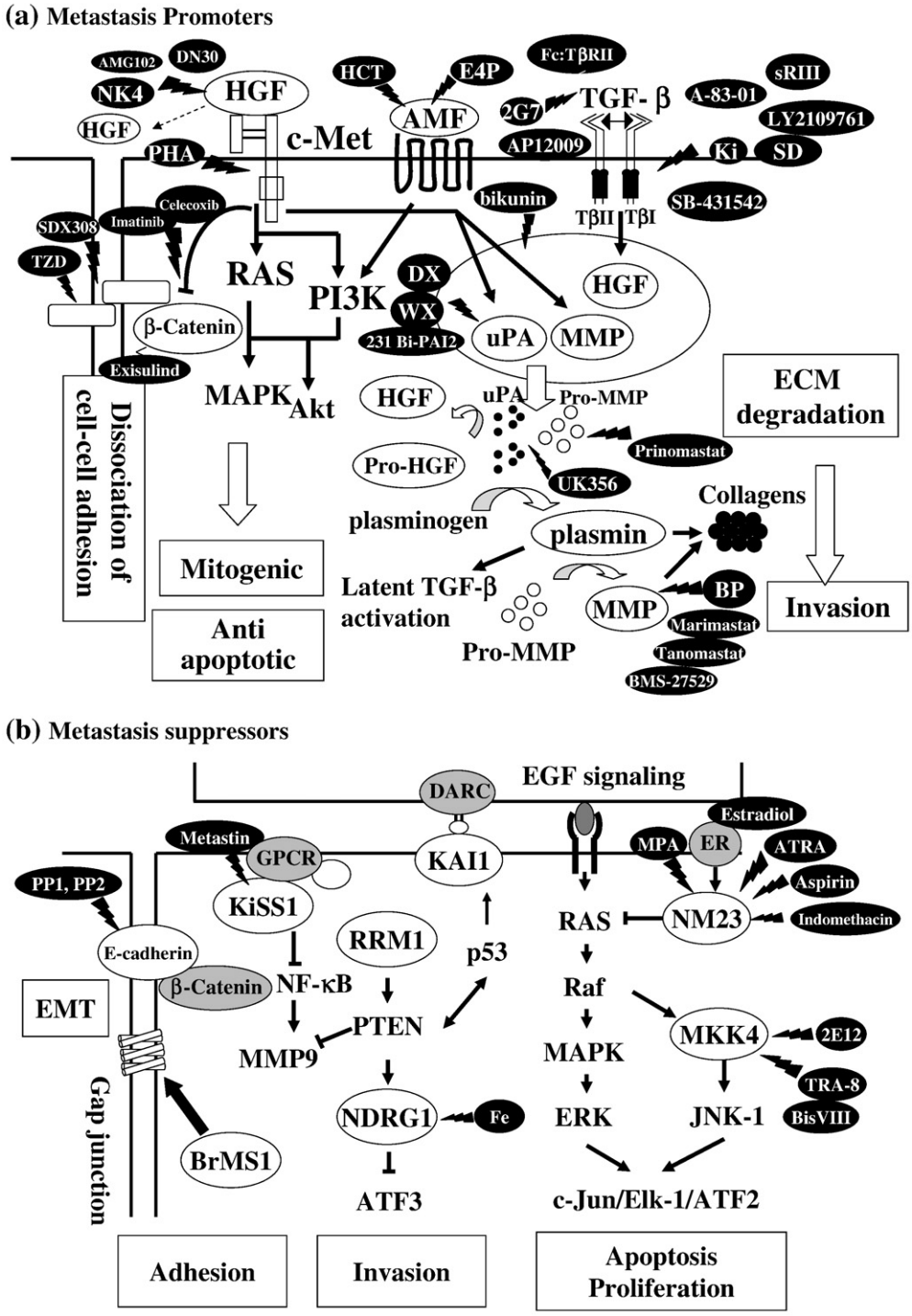

Fig. 2. Signal pathway of tumor metastasis. Tumor metastasis is a result of complex interplay of both positive (a) and negative (b) factors. These pathways and their factors are potential targets for anti-metastatic therapy. The drugs currently under development are shown as black oval shapes.

followed by significant inhibition of proliferation and invasion of various tumor cells lines both *in vitro* and *in vivo* [63]. Collectively, recent information about the mechanistic insight of HGF/c Met signaling in tumor progression has greatly facilitated the development of a variety of strategies for anti HGF/cMet therapies, and some of these compounds hold great promises for future clinical application.

3.3. Tgfβ

Transforming growth factor β (TGFβ) is a secreted polypeptide cytokine that plays multiple roles in cell proliferation, differentiation, extracellular matrix production, migration and apoptosis [64 66]. Notably, in normal epithelial cells and at an early stage of tumorigen esis, TGFβ inhibits the proliferation of cells by inducing cell cycle arrest, promoting apoptosis, and enhancing genomic stability [65,66]. However, as the tumor develops, cancer cells become resistant to TGF β mediated growth inhibition because of the loss of TGFβ signaling, mutations of cell cycle regulators, or alteration of cross talk signaling pathways such as activation of Ras [67].

TGFβ1 has been shown to be over expressed in 74% and 60% of patients with breast and colon cancers, respectively. Interestingly, more intense staining patterns for TGFβ1 are observed in various

types of metastatic cancer including breast, colon, liver, lung, prostate and stomach compared to primary tumors, emphasizing the importance of TGFβ signaling for pro metastatic activity [68]. Transplanting cell lines stably over expressing TGFβ1 into athymic mice has been shown to cause increased tumor growth and metastases *in vivo* [69,70]. In another study, transgenic mice that co express MMTV Neu and MMTV TGFβ1 developed mammary tumors with the same latency as the control MMTV Neu transgenic mice; however the co transgenics showed significantly more local invasion and elevated numbers of circulating tumor cells and lung metastases [71]. Thus, over expression of TGFβ can enhance and stimulate tumor growth and malignant progression at least in particular subtypes of tumors. Therefore, TGFβ has been recognized as a tumor promoter at an advanced stage of some tumors, probably by stimulating tumor cell invasion, angiogenesis and immunological surveillance [65,66].

It has been shown that mouse and human carcinomas often over express TGFβ, which promotes Epithelial mesenchyma Transition (EMT) via the Smad pathway [66]. Furthermore, Shen et al. have shown that TGFβ was capable of inducing the expression of guanine exchange factor NET1 via Smad3 followed by activation of the Rho GTPase pathway, which results in local disassembly of the actin cytoskeleton and tight junction breakdown [72]. On the other hand, TGFβ can also activate various non Smad signaling effectors including Ras, Rho GTPase, Erk1/2, PI3K and NF κB that all play critical roles in EMT, which eventually promotes tumor metastasis [67,73,74]. It has been shown that the motility of metastatic breast carcinoma cells responding to autocrine TGFβ1 did not require Smad activation but rather the activity of the PI3K pathway [74]. In addition, Vogelmann et al. have shown that in polarized epithelial cells, TGFβ blocked cell cell adhesion by inducing tyrosine phosphorylation of α and β Catenin which disrupts the E cadherin/catenin complexes with actin, and by inducing the expression of transcriptional repressors of the E cadherin gene such as Snail, Slug and LEF1 [75,76]. Wikstrom et al. showed that the ectopic expression of TGFβ in human prostate cancer correlated with increased angiogenesis around the tumor and eventually lead to a high rate of metastasis of prostate carcinoma cells [77]. The ability of TGFβ to promote angiogenesis is considered to be the action of either inducing expression of VEGF, which directly stimulates the proliferation and migration of endothelial cells, or its chemoattractant activity for monocytes that release angiogenic cytokines [78]. It should be also noted that, in breast cancer, TGFβ stimulates the expression of pTHrP (parathyroid hormone related protein) which promotes osteolytic metastasis and also suppresses late stages of osteoblast differentiation, which leads to net bone loss [79]. Furthermore, TGFβ plays a role in helping tumor cells to escape from the immunological surveillance through its ability to inhibit B and T lymphocyte proliferation and differentiation [80]. TGFβ is also able to deactivate macrophages and thus protect the tumor cells from the immune surveillance [81]. Collectively, because TGFβ often promotes tumor progression in particular subtypes, the components of the TGFβ signaling pathway are being considered as prognostic biomarkers for such tumors as well as potential therapeutic targets [68].

On the contrary, to the tumor promoting activity of TGFβ, this molecule also has tumor suppressive function at an early stage in some types of cancer. Therefore, TGFβ is considered as a target for chemoprevention for the population with high risk cancer incidence. To this end, several compounds have been examined and these include FTI 277, Dietary ω 3 fatty acids, Captopril, Suberoylanilide hydroxamic acid (SAHA) and triterpenoids. They are capable of enhancing the expression of TGF receptor (TβRII and TβRI) at mRNA and protein levels, thus increasing the responsiveness of tumor cells to TGFβ with respect to growth arrest and cytostatic effect [82 86]. However, considering the pro tumorigenic actions of TGFβ, such drugs may have dreadful effects by promoting tumor invasiveness and metastasis. Therefore, current effort is more focused on drugs that block the tumor progression at a later stage. These strategies include developing small molecule inhibitors, affinity or antibody based drugs and antisense RNA.

Intense high throughput screenings have led to the development of selective small molecule inhibitors against the enzymatic activity of the TβRII and TβRI kinases. These inhibitors including SD 208, SD 093, SB 431542, A 83 01 and LY2109761 act as ATP binding analogues and thus competitively block the catalytic pocket of the receptor kinase [68]. SD 208, an orally active specific TβRI kinase inhibitor, was previously tested in a glioma model, which depends primarily on the pro tumorigenic action of TGFβ. In this study, SD 208 was found to effectively inhibit the TGFβ induced glioma cell migration and invasiveness and also to enhance the immunological surveillance [87]. Recently, Reiss et al. also showed that SD 208 treatment resulted in decreased angiogenesis in a mouse model of mammary carcinoma [88]. In addition, Wong et al. showed that SD 208 reduced primary tumor growth and decreased the incidence of metastasis in an orthotopic xenograft mouse model of pancreatic adenocarcinoma [89]. Thus, this inhibitor holds a great promise for future clinical application. Another small molecule for TβRI kinase inhibitor, SD 093, has been shown to strongly decrease the *in vitro* motility and invasiveness of pancreatic carcinoma cells without affecting their growth [90]. Another set of TβRI inhibitors, SB 431542, A 83 01 and LY2109761, all potently affect TGFβ dependent transcriptional activa tion and inhibit TGFβ induced EMT [73]. Interestingly, SB 431542 was demonstrated to reduce colony formation of human lung adenocarci noma cells, which are growth dependent on TGFβ; however, it also induced anchorage independent growth of human colon adenocarci noma cells whose proliferation is promoted by TGFβ [91]. Further more, SB 431542 showed no effect on a cell line that failed to respond to TGFβ, which further strengthens the rationale in using this compound as a therapeutic agent of human cancer responsive to tumor promoting effects of TGFβ. A 83 01 is structurally similar to SB 431542 while it has shown even more potent effect of suppressing TβRI [73]. LY2109761 is a specific pharmacologic inhibitor of TβRI and TβRII kinases. It was demonstrated that this drug was capable of inducing the expression of the Coxsackie and adenovirus receptor (CAR), a tight junction component whose expression is required to be down regulated for EMT [92]. Currently, some of the above mentioned specific inhibitors of TβRI have already entered the phase I clinical trials for various human cancers (Table 1).

Neutralizing anti TGFβ antibodies and the soluble extracellular domain of TβRII with receptor binding activity have also been pursued as anti TGFβ approaches. Interestingly, the results of pre clinical studies have shown that these drugs had a weak and transiently negative effect on primary tumor growth but strongly suppressed metastasis [73]. Pietenpol et al. have demonstrated that the neutralizing antibody 2G7 which has high affinity to three mammalian isoforms of TGFβ showed moderate inhibitory effect on the growth of the primary tumor in an animal model of MDA MB 231 xenograft, while it almost completely blocked the abdominal and lung metastasis [69]. In addition, enforced expression of the extracellular domain of TβRII has been demonstrated to enhance tumor immune surveillance and strongly inhibit metastasis in animal models of human pancreatic carcinoma [93]. These observations led to a development of a fusion protein of immunoglobulin Fc fragment with the soluble extracellular domain of TβRII (Fc: TβRII) as a therapeutic approach [94]. When tested in vitro, this fusion protein indeed effectively induced apoptosis and inhibited migration of breast cancer cells. Furthermore, Wakefield et al. found that when Fc:TβRII was expressed in the mammary gland of MMTV based transgenic mouse model followed by a challenge of melanoma cells or by crossing it to the MMTV Neu mouse, it completely blocked lung metastasis without any adverse side effect [95]. The clinical potential of this experiment is significant especially because the chronic presence of

Fc:TβRII did not show obvious adverse effects. Similarly, Sun et al. have shown that over expression of soluble extracellular domain of β glycan (sRIII) antagonized TGFβ in the breast carcinoma cells, which resulted in significant inhibition of metastasis of the tumor cells to the lung, while it moderately blocked the tumorigenic ability [96].

Finally, the antisense DNA or RNAi technology have recently brought a promising development in anti TGFβ therapy. The oligo nucleotide AP12009, which is directed against human TGFβ2, has been tested by administering into brain tumors with continuous infusion and showed better survival time after recurrence than other current chemotherapy against gliomas [97]. Also, RNAi for both TGFβ1 and TGFβ2 in human glioblastoma has been reported to be effective in restoring the proper immune response, which significantly decreased the glioma cell motility and invasiveness [98]. Further investigations in this research field are expected to provide valuable information to improve the efficacy of these compounds and to develop a better delivery system for eventual clinical use of anti TGFβ therapy.

3.4. Mmp

Matrix metalloproteinases (MMPs), a group of zinc dependent endopeptidases, was originally identified to have roles in ECM disruption and thus associated with invasion and metastasis in late stages of cancer progression (Fig. 2A). Years of intense investigations of MMPs have highlighted the significance of these molecules in cancer. MMPs contribute to the formation of a complex microenvir onment that promotes malignant transformation in early stages of cancer, suppresses tumor cell apoptosis, and enhances angiogenesis as well as impairs the host immunological surveillance [99]. Several studies have indicated that cleavage of particular substrates such as insulin like growth factor binding proteins (IGFBPs) and TGFβ by MMPs can have direct effects on tumor growth [100,101]. In transgenic animals, over expression of certain MMPs such as MMP1 and MMP3 was sufficient to generate fully malignant tumors in the absence of specific carcinogens [102,103]. In the normal cells or at an early stage of tumor, MMPs can target substrates that influence the apoptotic process of the cells, which is also linked to the chemotherapeutic resistance. Particularly, MMP7 is able to release a soluble form of the death protein Fas Ligand (FasL), which has lower death promoting potency than the membrane anchored form but has more flexibility to interact with its cognate receptor Fas [104,105]. Thus, the weak but constant apoptotic signal acts as a selective pressure for tumor cells that have elevated anti apoptotic signals and those that have propensity to acquire additional mutations, which further promote tumor progression. This mechanism is also con sidered to be the basis of induction of chemoresistance to certain types of tumors [106].

MMPs also play critical roles in angiogenesis. Angiogenic factors such as basic fibroblast growth factor (bFGF) and VEGF are usually localized in the matrix and cannot interact with their receptors until freed by MMPs, particularly by MMP9 through ECM proteolysis [107,108]. In addition, MMP9, when recruited to the tumor cell surface and interact with the docking receptor CD44, can proteolytically cleave latent TGFβ and thus promote tumor invasion and angiogenesis [100]. Furthermore, an elegant work of Hanahan and Coussens has shown that MMP9 is predominantly expressed in the tumor associated stromal cells as well as in macrophages, neutrophils, mast cells and endothelial cells rather than in tumor cells themselves in many cases, which regulates the vascular formation and architec ture [109 111]. Intriguingly, Hiratsuka and colleagues have recently shown that MMP9 plays a role in priming premetastatic sites for primary tumor. They demonstrated that tumor associated macro phages (TAM) induced MMP9 in endothelial cells and in TAMs, which facilitated tumor cell invasion and also prepared the lung as premetastatic niche for the growth of tumor cells in a manner dependent on VEGFR 1 [112].

Escaping from host immune response is a significant problem associated with many cancers. Some MMPs alter the behavior of chemokines and cytokines by specific proteolytic cleavage. For example, MMP9 can suppress the development and propagation of T lymphocytes by disrupting IL 2Rα signaling, resulting in attenuation of a T cell mediated anti tumor response [113]. Likewise, CXCL12, also known as SDF1 has been identified as a substrate of MMP2. MMP2 mediated cleavage renders CXCL12 unable to bind its receptor CXCR4, which consequently influence the metastatic dissemination of tumor cells [114].

The strong correlations between altered expression of MMPs at mRNA and protein levels in different human cancers with poor disease prognosis have been well established [99,115]. The over expression of many MMPs, including MMP 1, 2, 7, 9, 13, 14, is positively associated with tumor progression and metastasis [115]. On the other hand, human breast tumor cells with reduced expression of MMP 8 were found to acquire the metastatic ability compared to their non metastatic counterpart [116]. Interestingly, Balbín et al. has revealed that MMP8 null mice exhibit an increased tumor susceptibility compared to the wild type because of the attenuation of adaptive immune responses due to the loss of MMP8 [117]. Similarly, MMP 3 knockout mice exhibited increased rate of initial skin tumor growth [118]. However, altered expression pattern or levels of individual MMPs in tumor or stromal cells do not always correlate in the primary tumors and secondary metastatic sites [115]. Interestingly, over expression of MMPs is frequently accompanied with a corresponding increased expression of natural inhibitors (TIMPs) of MMPs, which result in reduced tumorigenesis in some model systems but does not necessarily inhibit metastasis [119,120]. These discrepancies point out the complexity of MMP functions *in vivo*.

The link between MMPs activity and malignant progression has stimulated serious effort in developing pharmacological inhibitors of MMPs (known as MMPIs) as a potential therapeutic modality since the 1980s [121]. A variety of MMP inhibitors including Marimastat (BB 2516), Prinomastat (AG3340), Tanomastat(BAY12 9566) and BMS 275291 Neovastat were found to be orally active and achieved effective blood levels and displayed high specificity to MMPs while sparing most other types of proteases [122]. These MMPIs have been shown to be effective in controlling cancer progression in animals. However, most clinical trials have come to a crashing halt with the repeated failure in multiple large scale phase III stage [122]. Even worse, some compounds caused severe side effects such as inflam mation, musculoskeletal pain and joint stricture [122]. Considering the ability of MMPs to cleave not only ECM but also a variety of other factors, cytokine precursors and chemokines, it may not be surprising to see unwanted chaotic immune responses. Therefore, this area of research requires newer strategies.

A recent work of Taketo and colleagues has provided valuable insights regarding a possibility of targeting the MMP producing cell instead of inhibiting MMPs themselves [123]. They found that immature myeloid cells expressing CC chemokine receptor (CCR1), MMP2 and MMP9 infiltrated the tumor invasion front and migrated toward the CCR1 ligand CCL9, whereas blocking CCR1 expression resulted in the accumulation of MMP expressing cells at the invasion front and suppressed tumor invasion in an animal model. Although an application of this “cellular target” concept is still premature and is waiting to be confirmed by multiple studies, it is expected to cause fewer side effects than the systemic “molecular target” therapy using MMP inhibitors. One important lesson we learned from the past clinical trials of MMPs inhibitors is the need for attention to the stage and type of cancer and the critical selectivity of MMPs inhibitors since the expression pattern of MMPs varies in various cancer types and stages [122]. For example, small cell lung cancer is known to over express MMP11 and MMP14 rather than MMP2, thus the MMP2 specific inhibitors like Tanomastat and Prinomastat would lead to a poor outcome [124]. One possible strategy is to take advantage of both

the frequent over expression of MMPs in malignant tumors and the catalytic functions of these enzymes, and this strategy led to the development of protease activatable retroviral vectors, which contain engineered MMP cleavable linkers [125,126]. Another approach is to employ macromolecular carriers that are linked to anti cancer drugs or immune response stimulating drugs that can be released from its carrier when encountered with MMPs in the tumor environment [127,128]. Alternatively, designing an inhibitor which targets sub strate specific binding sites of MMPs resulting in reduced binding and cleavage of specific substrates of the corresponding MMP opened a possibility of blocking the unwanted catalytic activity of MMPs during tumor progression [99]. Finally, re screening for MMPs inhibitors from the current anti cancer drug pool may be worth a consideration. Notably, Bisphosphonates (BP), a class of pyrophosphate analogues widely used in the treatment of breast cancer patients with osteolytic tumors for the past 20 years, was found to significantly inhibit proteolytic activity of MMPs without reducing the expression of MMPs [129]. Although past efforts in developing anti MMP drugs have been less fruitful than expected, there are still strong rationales and hopes to continue this line of research using more innovative approaches.

3.5. Upa

The urinary type plasminogen activator (uPA) is a serine protease and able to proteolytically degrade various ECM components and the basement membrane around the primary tumors. It also activates multiple growth factors and MMPs that further contribute to the degradation of the ECM, and thus facilitates tumor cell invasion and intravasation (Fig. 2) [130,131]. Interestingly, a newly identified metastasis suppressor, p75 neurotrophin receptor ($p75^{NTR}$), has recently been demonstrated to suppress metastasis in part by down regulating specific proteases such as uPA [132]. uPA is produced and secreted as a zymogen (pro uPA) which binds to the cell surface uPA receptor, uPAR. The pro uPA is then cleaved by plasmin to become an active form of uPA, which has plasminogen activating property to convert plasminogen to the active matrix degrading serine protease plasmin [131]. The proteolytic activity of uPA is regulated by the serine protease inhibitors, plasminogen activator inhibitor 1 (PAI 1) and PAI 2. PAI 1 is able to react with uPA/uPAR complex and induces internalization of the complex, which results in the intracellular degradation of uPA and PAI 1. On the other hand, PAI 2 forms a complex with uPA and uPAR without internalization, and it is degraded once bound to uPA/uPAR [133]. Because the activity of uPA is dependent on its binding to uPAR, this receptor is also considered to play a crucial role in metastasis [130]. Besides the role in proteolysis, uPAR can interact with and regulate other cell surface proteins such as integrins, growth factor receptors and G protein coupled receptors to exert its biological functions including chemotaxis, cell migration and invasion, adhesion, proliferation and angiogenesis [134].

Several recent studies have shown that uPAR is also involved in activation of the signaling of other metastasis promoting factors such as basic fibroblast growth factor (bFGF), VEGF, TGFβ and HGF (Fig. 2) [130,135,136]. Most normal tissues have little or no detectable uPAR, while uPAR is over expressed across a variety of carcinomas including colon, breast, ovary, lung, kidney, liver, stomach, bladder, endometrium and bone [131,137,138]. uPAR expression has also been shown to be strongly correlated with advanced metastatic cancer, and it is typically found to be abundant at the invasive boundary between tumor cells and normal tissue [139,140]. This localization of uPAR expression in the invasion front may be due to the fact that uPAR is a hypoxia inducible gene [141,142]. Importantly, the uPAR expression has been found to correlate with a poor prognosis and mortality of patients with various types of solid tumors [141 143]. Currently, the PAI 1 is considered as one of the most informative prognostic markers in several cancer types and a high PAI 1 level is significantly associated with a poor prognosis in these cancers [144 147]. The precise role of PAI 1 in tumor growth and metastasis is yet to be elucidated, but PAI 1 shows diverse functions depending on the cell context and the expression level [148]. Interestingly, several reports indicated that unlike PAI 1, PAI 2 functions as a tumor suppressor and blocks metastasis, and therefore, is associated with a favorable outcome in patients [143,149]. In addition, uPA and PAI 1 have also been reported to be associated with resistance to hormone therapy in advanced breast cancer [150]. Therefore, uPA/PAI 1 can also be used to predict resistance to specific therapies for breast cancer patients. These studies of uPA/uPAR and PAI 1 so far indicate the critical roles of these molecules in tumor progression, suggesting that these proteins serve as excellent therapeutic targets for cancer patients.

In the past, various approaches have been developed to inhibit uPA and its signals. WX UK1 and WX 671, synthetic serine protease inhibitors developed by WILEX, are the first inhibitors of uPA in world wide clinical trials. Both of them have shown to effectively block metastasis formation and to reduce primary tumor growth in pre clinical studies, and they have already entered the phase I/II clinical trials as a single agent and/or in combination with other chemother apeutics for the treatment of patients with metastatic tumors [148]. Bikunin, a Kunitz type protease inhibitor, is discovered as a potent and selective inhibitor for trypsin and plasmin, while it is moderately effective in inhibiting the catalytic activity of uPA [151]. Kobayashi et al. have also shown that Bikunin was able to down regulate the expression of uPA and uPAR [152]. Furthermore, Bikunin has been shown to inhibit MAPK and PI3K/Akt signaling, and to effectively inhibit growth and invasiveness of several types of tumor cells [153 155]. Recently, the possibility of using Bikunin as oral therapy was examined in an ovarian cancer model in animal. Results of these experiments have shown that once daily oral administration of Bikunin had no significant side effects and strongly suppressed the expression of uPA and uPAR, suggesting a utility of Bikunin for an anti metastatic therapy in humans [156].

DX 1000, another Kunitz domain based inhibitor of plasmin with specificity, has been previously shown to block tumor growth and metastases *in vivo* with few side effects [157] However, DX 1000 has a quick clearance and short half life in circulation that challenges the practical utility of this compound in patients. To circumvent these problems, Henderikx et al. conjugated the DX 1000 with polyethyle neglycol (PEG) to prolong *in vivo* half life. The PEG conjugated DX 1000 was indeed shown to be effective *in vitro* and significantly blocked tumor proliferation, vascularization and metastasis *in vivo* [158]. More recently, Fishe et al. have shown that 1 Isoquinolinylgua nidines (UK 356,202) and its derivatives were able to reversibly inhibit uPA enzymatic activity with selectivity over tPA and plasmin, and it has been selected as a candidate for clinical evaluation [159]. There are also several other strategies currently under active investigation and these include receptor ligand analogues to interfere with the cellular uPA/uPAR interaction, antibodies for PAI 1 and recombinant PAI 2 (231Bi PAI2) [160 162].

3.6. β catenin

β Catenin is an essential component of the cadherin catenin complex and plays a critical role in the Wnt signaling pathway [163]. The product of the tumor suppressor gene APC (adenomatous polyposis coli) forms a complex with axin/axil, protein phosphatase 2A (PP2A) and glycogen synthase kinase3β (GSK3β) which leads to phosphorylation of β Catenin thereby inducing degradation of this protein by ubiquitination mediated proteasomes [164]. The abnor mally activated Wnt signaling due to the mutations of APC results in accumulation of β Catenin followed by promotion of tumorigenesis. Phosphorylation of β Catenin also releases E cadherin, which initiates tumor cell migration and tumor metastasis [165,166]. On the other

hand, β Catenin together with other proteins such as TCF/LEF complex, Reptin and p50, acts as a transcription factor to regulate metastasis related gene including MMP 9 and KAI1 [167]. More recently, it has been reported that accumulated β Catenin binds specifically to androgen receptor (AR) and augments the ligand independent activity of AR in hormone refractory prostate cancer [168]. Indeed, aberrant expression of β Catenin has been reported in many types of cancer including colon, bladder, breast, prostate, lung cancer and adrenocortical adenomas [169]. Furthermore, the Wnt/β Catenin signaling pathway has been shown to be involved in the self renewal of embryonic stem cells and perhaps in progression of tumor stem cells [170]. Several agents targeting the Wnt/β Catenin pathway including Exisulind and Imatinib have been shown to inhibit self renewal of cancer stem cells with varying levels of success [171]. Therefore, targeting β Catenin and blocking APC/β Catenin/TCF signals is considered to be a rational approach for developing new anti cancer drugs.

Exisulind (Aptosyn) and two analogs CP461, CP248 belong to a new class of compounds of SAANDs (Selective Apoptotic Antineoplastic Drugs), which are oxidative metabolites of the nonsteroidal anti inflammatory drug (NSAID) sulindac. These drugs reduce β Catenin activity and block Cyclin D1 followed by an induction of apoptosis and inhibition of tumor cell growth [164,172,173]. Currently, Exisulind is in Phase III clinical trials in combination with several chemotherapeutic agents [174,175]. Imatinib (Gleevec), originally identified as an inhibitor of platelet derived growth factor (PDGF) receptor, has been used in treating chronic myelogenous leukemia (CML), gastrointest inal stromal tumors (GISTs) and a number of other malignancies. Interestingly, Imatinib has been shown to inhibit tyrosine phosphor ylation of β Catenin, which otherwise releases E cadherin and promotes cell migration and tumor metastasis [176]. Other strategies including RNAi, antisenseDNA and small molecule inhibitors for blocking β Catenin have been developed [171,177]. The antisense approach has been used in colon and esophageal cancers as well as leukemia and lymphoma *in vitro*, which lead to reduction of β Catenin expression and subsequent decrease in the expression of its down stream targets such as Cyclin D1 [177 179].

NSAIDS are also found to be effective in inhibiting the Wnt/β Catenin signaling pathway. Among them, aspirin and indomethacin were shown to block the transcriptional activity of β Catenin/TCF [180]. Celecoxib (a COX 2 inhibitor) blocked β Catenin activity by inducing its degradation via GSK3 β and APC, leading to diminished tumor cell proliferation and survival [181]. R Etodolac (an enantiomer of Etodolac) and its analog (SDX 308) have been shown to be able to decrease total and activated forms of β Catenin via GSK3 β activation [182]. These drugs also increased β Catenin and E cadherin complex at the membrane site and inhibited β Catenin dependent TCF activity followed by decreasing the level of downstream target gene products, Cyclin D1 and glutamine synthetase [183,184]. In addition to these efforts of directly blocking the β Catenin activity, selective disruption of β Catenin TCF complex and reversing the localization of β Catenin from cytoplasmic membrane to the nucleus are also considered to be effective approaches for anti cancer therapy. Thiazolidinedione (TZD), a peroxisome proliferator activated receptor gamma ligand, has been demonstrated to completely inhibit lymph node and lung metastases in a xenograft animal model by promoting localization shift of β Catenin from the nucleus to plasma membrane [185]. TZD also reduced tyrosine phosphorylation of β Catenin and promoted enhanced expression of E cadherin [185]. Recently, a crystal structure of β Catenin TCF complex has been clarified which shed new light on the molecular mechanism by which this stable and potent transcrip tion factor complex forms [186 188]. Therefore, developing a drug which can disrupt the β Catenin TCF complex holds great promise, although how to effectively and selectively disrupt the complex without affecting β Catenin E cadherin or APC complex is still a challenge.

4. Metastasis suppressors

4.1. Nm23

NM23 is the first identified metastasis suppressor gene in this group. It is located on chromosome 17q21 and codes for an 18.5 kDa protein containing 166 amino acids which functions as nucleoside diphosphate kinase and protein histidine kinase [189,190]. Clinically, NM23 has been shown to be down regulated in a variety of tumors including breast and prostate cancers [191,192]. Ectopic expression of NM23 has also been shown to significantly reduce the *in vitro* and *in vivo* metastatic potential of highly metastatic carcinoma cell lines including breast, melanoma, colon, and oral squamous cells [190,193 195]. Recently, Hartsough et al. reported that NM23 formed a complex with Kinase suppressor of Ras1 (KSR1) and phosphorylated this protein at Ser 392 and Ser 434, which resulted in blockade of Ras/MAPK pathway (Fig. 2b) [196]. More recently, Salerno et al. have shown that the NM23 expression level influenced the binding properties, stability and function of the KSR1 in breast carcinoma cells [197]. Hence, NM23 was hypothesized to inhibit MAPK/ERK activation via altering the scaffold function of KSR1 (Fig. 2b). Consistent with this hypothesis, MDA MB 435 breast cancer cells that over express NM23 showed reduced MAP kinase activity and cell motility *in vitro* as well as diminished incidence of metastasis *in vivo* [196,198,199]. Therefore, NM23 acts as a metastasis suppressor by inhibiting the MAP kinase pathway through the interaction with the KSR1 scaffold protein.

In an attempt to restore the expression of NM23 in tumor cells, several drugs have been found in the past. Among them, medroxy progesterone acetate (MPA) and estradiol were reported to suppress metastasis through up regulation of the NM23 gene (Table 1). Medroxyprogesterone is a progestin and commonly used as a component of hormonal contraceptives. Progesterone binds to the progesterone receptor which is then transferred to the nucleus and acts as a transcription factor by binding to the progesterone response elements (PRE) in the promoter region of target genes. Progesterone receptor is known to directly regulate the expression of Cyclin D1, beta casein and $p21^{WAF1}$ as well as MAPK [200 205]. MPA has a long history of clinical use at a low dose as the contraceptive Depo Provera and has also been used for hormone replacement therapy in combination with estrogen [206]. At a high concentration, it has been used for the treatment of advanced breast and endometrial cancers [207]. MPA can competitively bind to several steroid hormones including progesterone (PR), androgen (AR) and gluco corticoids (GR), and thus it is able to up regulate NM23 by antagonizing the effect of glucocorticoid response element (GRE) on the NM23 promoter [208]. Ouatas et al. previously found that MPA inhibited the soft agar colonization of breast carcinoma cells by up regulating the NM23 expression [209]. In *in vivo*, Palmieri et al treated mice xenografted with breast carcinoma cells with MPA and found 27 36% reduction of metastasis incidence in the treated animals.

Estradiol works as an estrogen to modulate gene expression via binding to its intracellular receptor ERs [210]. Interestingly, Estradiol was found to be able to decrease the number of experimental lung metastases in nude mice when they were injected with breast cancer cell line MDA MB231 with forced expression of ER (Table 1) [211]. Lin et al. reported that the level of NM23 mRNA and protein was induced by Estradiol in breast cancer cell lines with the extent that these effects correlated with the level of ERα expression [212]. In addition, Estradiol was shown to be able to decrease the invasive ability of ERα positive carcinoma cell lines MCF7 and BT 474, while it did not have any effect on BCM 1 cell which had virtually no ERα expression [212]. Therefore, it is suggested that Estradiol was able to suppress tumor metastasis by activating the expression of the NM23 gene in an ERα dependent manner (Fig. 2b) [212].

Many of the therapeutic effects of nonsteroidal anti inflammatory agent (NSAIDs) are clearly due to the inhibition of prostaglandin synthesis by inactivation of cyclooxygenase 1 and 2 (COX 1 and COX 2) [213]. The anti tumor effect of NSAID has been recognized when Aspirin was found to reduce the risk of colorectal adenoma and carcinoma in animal models [214 217]. Interestingly, Yu et al. reported that Aspirin decreased the invasive potential of COX2 negative colon cancer cells via up regulation of NM23 expression (Table 1) [217].

Another NSAID, Indomethacin, was also found to up regulate the expression of NM23 in breast cancer cells and to alter the malignant choline phospholipid phenotype toward a less malignant tumor [218]. Reich et al. reported that indomethacin reduced the invasive ability of human fibrosarcoma and murine melanoma cell lines and that murine melanoma cells exposed to indomethacin prior to i.v. injection produced significantly fewer lung metastases (Table 1) [219]. Kundu et al. also reported the anti metastasis effect of indomethacin by oral administration in a murine model [220]. They transplanted a murine mammary adenocarcinoma cell line 410.4 and found that the metastatic ability of this cell line was reduced by almost 50% with the treatment of indomethacin (Table 1) [220]. Therefore, indometha cin has potential utility as an anti metastatic drug and it is currently under clinical trial.

All *trans* Retinoic Acid (ATRA) is known as the first successful targeted drug for cancer therapy. ATRA causes the differentiation of leukemic myeloid cells from mature myeloid cells by attaching to one of several retinoid receptors in the cell nucleus and then directly modulating gene expression [221 223]. The down regulation of several oncogenes including Ras and c fms by ATRA has been reported [224,225]. Interestingly, the expression of NM23 was also shown to be up regulated by ATRA in human hepatocarcinoma cell line and gastric cancer cell lines [226,227]. Liu et al. demonstrated that treatment with either ATRA or transfected NM23 cDNA reduced metastasis associated phenotypes including chemotaxic cell migration and invasion of human hepatocarcinoma cell line [226]. Furthermore, Wu et al. examined the effect of ATRA treatment in xenografted nude mice and found that ATRA treatment significantly decreased the metastasis in liver and increased NM23 protein levels in experimental groups compared with a control group [227]. Since ATRA was also able to reduce cell growth *in vitro* and *in vivo* [227], the specificity of ATRA treatment on tumor metastasis is still unclear. However, a combina tion treatment of ATRA and IFN alpha in a clinical trial was well tolerated, and patients who have metastatic osteosarcoma were found to be in stable complete remission 14 months after the end of therapy [228]. Therefore, further investigation of ATRA as an anti metastatic drug is warranted.

4.2. KiSS 1

KiSS 1 was originally identified as a metastasis suppressor gene using a combined strategy of MMCT and differential display [229]. The introduction of an intact copy of whole human chromosome 6 into the C8161 human melanoma cell resulted in significant reduction of metastasis ability of this cell line without affecting tumorigenicity or local invasiveness in animals [229]. Later Lee et al. reported that the KiSS 1 gene was actually mapped on chromosome 1q region which is frequently deleted in late stage human breast carcinomas [230]. They then transfected the KiSS 1 gene into human breast ductal carcinoma cell line MDA MB 435 and found that KiSS 1 almost completely suppressed metastatic activity of MDA MB 435 [230]. Therefore, although the KiSS 1 gene is located on chromosome 1, it is believed that chromosome 6 is responsible at least in part for its metastasis suppressive effects by harboring a gene that positively regulates KiSS 1 expression [231]. Clinically, the expression of mRNA of the KiSS 1 gene was found to be significantly down regulated in metastatic tumors, which is in accordance with the idea that KiSS 1 is a metastasis suppressor [232].

Ectopic expression of the KiSS 1 gene was shown to significantly reduce the rate of three dimensional growth in soft agar, but it did not affect invasion or motility [230]. These results suggest that KiSS 1 affects downstream of cell matrix adhesion and perhaps involves cytoskeletal reorganization. On the other hand, Yan et al. reported that KiSS 1 transfected HT1080 cells showed substantially reduced enzyme activity of MMP9 with specific down regulation of mRNA level of MMP9 and invasiveness of tumor cells *in vitro* [233]. They have further shown that this effect was partly attributable to the ability of KiSS 1 to reduce NF kB binding to the promoter of MMP9 by enhancing I kB activity (Fig. 2b) [233].

Metastin is a 54 amino acid peptide whose sequence is identical to a part of the KiSS 1 gene, and this peptide was found to act as a ligand for orphan G protein coupled receptor (hOT7T175, AXOR12, GPR54) (Table 1) [234,235]. Interestingly, Ohtaki et al. have shown that Metastin significantly attenuated pulmonary metastasis in a mouse xenograft model using the B16 BL6MR melanoma cell, while Metastin had no direct effect on the primary tumor growth [234]. Importantly, Metastin was found to be able to suppress the degree of pulmonary metastasis even when the peptide was administered to the mice that already had metastasis in the lung [234]. Therefore, Metastin is considered to be a promising agent for the treatment of metastatic cancer patients. In this regard, it is encouraging that the expression of the Metastin receptor genes was found to be normal even when KiSS 1 was significantly down regulated in various types of cancers [236]. These results suggest that Metastin may be effective even in advanced cancer that has lost KiSS 1 expression.

4.3. Mkk4

Chekmareva et al. has previously demonstrated a prostate cancer metastasis suppressor activity encoded by a discontinuous ~70 cM region of human chromosome 17, which suppresses the spontaneous metastatic ability of highly metastatic Dunning AT6.1 rat prostate cancer cells [237]. Later, Yoshida et al. identified the MKK4/SEK1 (Mitogen activated protein kinase kinase 4) gene in this chromosomal region as a candidate metastasis suppressor [238]. Ectopic expression of MKK4 in highly metastatic prostate cancer cell line indeed significantly suppressed macroscopic lung metastasis without affecting the primary tumor growth in animals [238]. Furthermore, Kim et al. examined the status of MKK4 expression in clinical samples of prostate cancer by immunohistochemical analysis and found that the expression of MKK4 was inversely correlated with Gleason score and tumor progression [239]. How MKK4 suppresses metastasis is a crucial question and has been under active investigation. MKK4 belongs to MAP kinase family which plays central roles in cell proliferation, differentiation and apoptosis. It is known that MKK4 is activated in response to a variety of extracellular stimuli including stress followed by activation of JNK(c Jun N terminal kinase) and/or p38 MAPK pathways (Fig. 2b) [240]. It is plausible that, when a tumor cell reaches a distant organ site, the expression of MKK gene in cancer cell is suppressed in the stressful environment, and therefore, fails to establish colonization.

A strategy of using monoclonal antibodies has been considered to be an attractive approach for cancer therapy due to their high target specificity. Anti death receptor antibody such as anti TRAIL anti bodies, 2E12 and TRA 8, have been found to activate the MKK4/JNK/p38 pathway, suggesting a potential utility of the antibodies for anti metastatic therapy [241]. Furthermore, Ohtsuka et al. reported that the combination of the anti death receptor antibodies and che motherapy agents led to a synergistical activation of the JNK/p38 MAP kinase which was mediated by MKK4 (Table 1) [241]. In their studies, agonistic anti TRAIL antibodies 2E12 and TRA 8, when combined with chemotherapeutic agents such as Adriamycin, were able to increase the release of cytochrome c and Smac/DIABLO from mitochondria in parallel with the profound loss of mitochondrial membrane potential, which resulted in apoptosis in breast, prostate and colon cancer cells

[241]. It is interesting to test whether these regimens are able to suppress metastatic potential of MKK positive cancer cells *in vivo*. Bisindolylmaleimide VIII was originally developed as a synthetic inhibitor of protein kinase C (PKC) [242,243], and it was later found to promote Fas mediated apoptosis in a PKC independent manner [244]. Ohtsuka et al. examined a possible effect of Bisindolylmaleimide VIII on TRA 8 induced apoptosis and found that a combination of Bisindolylmaleimide VIII and TRA 8 induced 50 80% of apoptosis in human astrocytoma cell line (1321N1), while the treatment of the cells with TRA 8 alone induced apoptosis only in up to 20% of the cells [245]. In *in vivo*, either Bisindolylmaleimide VIII or TRA 8 alone partially regressed the xenografted tumor in NOD/SCID mice, while the combination of these two drugs almost completely blocked the tumor growth. However, whether Bisindolylmaleimide VIII enhances TRA 8 induced apoptosis via a role in regulating MKK4/JNK/p38 apoptosis kinase signaling and whether the combination of these drugs indeed suppresses metastasis remains to be examined.

4.4. E cadherin

The transmembrane protein E cadherin (also known as CDH 1) was originally isolated as human uvomorulin by screening a cDNA library of the human liver [246]. The E cadherin is a calcium dependent adhesion molecule which constitutes the adherence junction in epithelial cells [247,248]. Reduced level of E cadherin is shown in a variety of human cancers at advanced stages. It is believed that a low level of E cadherin can give advantage to tumor cells on breaking the adhesion junction and detaching from adjacent cells, so that these cells invade and metastasize to other distant organs. Clinically, several groups have reported that decreased expression of E cadherin was associated with a poor prognosis in cancer patients [249]. On the other hand, over expression of E cadherin in invasive cancer cells has been shown to decrease motility and invasiveness [250]. In addition, using a transgenic mouse model of pancreatic β cell carcinogenesis (Rip1Tag), Perl et al. showed that tumor incidence or tumor volume was not significantly changed between double transgenic Rip1Tag2xRip1dnE cad mice and single transgenic Rip1Tag2 littermates [251]. However, the double transgenic mouse developed metastases to the pancreatic lymph nodes, an invasive phenotype that was never observed in single transgenic Rip1Tag2 mice [251]. Therefore, E cadherin is considered to function as a metastasis suppressor. Generally, E cadherin plays an important role in epithelial mesenchymal transition (EMT) during which epithelial cells lose their cell cell junctions and acquire mesenchymal characteristics to endow the migratory ability to tumor cells [249]. E cadherin interacts with β Catenin to mediate actin binding (Fig. 2b) [252]. Therefore, loss of E cadherin, in addition to reducing cell cell adhesion, provides an oncogenic stimulus by freeing β Catenin from the membrane, so that β Catenin can travel to the nucleus to activate TCF regulated genes such as c Myc and Cyclin D1 [253]. Furthermore, E cadherin has been recently found to be down regulated by transcription factors Snail and Slug that are involved in the process of EMT, cell differentiation and apoptosis [254]. Therefore, restoring the function of E cadherin is considered to be a potential therapeutic option for metastatic disease. PP (pyrazolo [3,4 d] pyrimidines)1and PP2 were originally identified as selective inhibitors for Src, and they were shown to be able to block tumor growth and to reduce metastasis in a mouse pancreatic model. However, these compounds have also been found to reactivate the E cadherin expression in pancreatic and colon cancer cells (Table 1) [255,256]. Therefore, PP1 and PP2 may serve as effective anti metastatic drugs although they need to be tested more extensively in a clinical trial.

4.5. Ndrg1

N myc downstream regulated gene 1 (NDRG1) was originally identified by differential displays as being significantly up regulated by induction of *in vitro* differentiation of colon carcinoma cells [257]. The protein encoded by the NDRG1 gene has a molecular weight of 43 kDa and possesses three unique 10 amino acids tandem repeats at the C terminal , among which seven or more phosphorylation sites were predicted and later they were shown to be targets of protein kinase A *in vitro* [258]. The NDRG1 gene is controlled by multiple factors and responsive to various stimuli. The expression of NDRG1 was repressed by C myc and N myc/Max complex *in vitro*, while it was induced by p53, hypoxia and PTEN (Fig. 2b) [259]. NDRG1 has been shown to act as a tumor suppressor as well as a tumor metastasis suppressor depending on cell context [259]. In a clinical setting, NDRG1 was found to be consistently expressed in normal prostate tissue as well as PIN (prostatic intraepithelial neoplasia) and BPH (benign prostatic hyperplasia), whereas the expression was significantly reduced in high grade tumors [260,261]. In addition, the level of the NDRG1 expression was inversely co related with the status of metastasis in these patients, supporting the notion that NDRG1 is a tumor metastasis suppressor [260]. In breast cancer, a similar and significant negative correlation of NDRG1 with metastasis has been observed, while the expression of NDRG1 does not show any significant correlation with the size or the histological grade of the primary tumor [261]. These results strongly suggest the negative involvement of NDRG1 in the process of invasion and metastasis in both prostate and breast cancer. Furthermore, ectopic expression of the NDRG1 gene in a highly metastastic prostate cancer cell line significantly reduced the incidence of lung metastases, suggesting that NDRG1 was able to block the metastatic process without affecting the primary tumor growth [260,261]. Similar metastasis suppressor effect of NDRG1 was also observed in colon carcinoma cells by Guan et al. [262]. In addition, NDRG1 also significantly suppressed the invasive potential of prostate and breast cancer cells as tested by *in vitro* invasion chamber assay [260,261]. Therefore, evidence from both clinical data and the results of *in vitro* as well as animal experiments overwhelmingly support the notion that NDRG1 is a metastasis suppressor gene and that the down regulation of the gene results in acceleration of tumor metastasis. How NDRG1 suppresses the tumor metastasis is an intriguing question which is under active investigation.

Recently, Fe chelators, desferrioxamine (DFO) and 311 were shown to be able to up regulate the NDRG1 expression in human breast cancer cell line MCF7 [263]. In the past years, dietary Fe restriction has been shown to markedly decrease tumor growth in rodents [264 266], and Fe chelators such as Triapine and desferrioxamine (DFO) were reported to be potentially useful for cancer therapy (Table 1) [266 268]. More recently, Whitnall et al. examined the effect of another Fe chelator, di 2 pyridylketone 4,4, dimethyl 3 thiosemicarbazone (Dp44mT), on tumorigenesis in xenografted mice models of lung carcinoma, neuroepithelioma and melanoma and found that Dp44mT strongly inhibited the growth of all tested human xenografts in nude mice [269]. Notably, Dp44mT significantly augmented the expression of the NDRG1 gene in the tumor compared to that of control group, suggesting a promising utility of this compound as an anti cancer as well as anti metastatic drug [269].

5. Conclusion and future direction

Despite significant improvement in surgical techniques and chemotherapy for cancer treatment in general, none of the current medical technologies “cure” the metastatic disease, and the patients who have already acquired metastatic cancer are left virtually with no options. Therefore, there is an urgent need for developing a novel approach of target specific therapy to metastatic tumor cells, which requires more comprehensive understanding of the molecular mechanism of metastases. The goals of anti metastatic therapy are three folds. Firstly, we need to develop a specific drug that blocks secondary metastasis to treat patients who have already acquired metastatic disease but are still at an early stage. Secondly, a drug

should also be developed to treat patients who underwent surgical resection of their primary tumors in order to prevent a possible recurrent disease. However, the ultimate goal is to develop a non toxic agent which can be taken as diet for prevention of metastasis. In the past decade, the major effort of anti cancer research has been focused on the development of drugs that can block the proliferation of tumor cells. They take advantage of the fact that tumor cells are more actively proliferating than other normal cells, and therefore, "selectively" kill the cancer cells. However, this "selectivity" has narrow margins and these agents inevitably cause severe side effects even when they are used in combination to lower the toxicity. From these experiences, we have learned an important lesson that the most critical issue for anti cancer drugs is their specificities. Therefore, to develop an anti metastatic drug, it is crucial to define a target molecule which is specifically expressed in metastatic cells. Ideally, an agent which can attack the molecule is inactive (pro drug) when given to patients, and is activated only in the tumor cells. In theory, monoclonal antibodies and siRNA are highly specific to target genes, and active investigations are underway to utilize these technologies for the development of anti metastatic drugs. If a target is well defined and specific, these agents are considered to be very effective, although there are still many unknown technical questions such as stability and delivery method of these agents. However, recent advance ment of bio technology such as nano particles has provided us with a hope that we can eventually overcome these problems.

We have learned a great deal of the metastasis cascade, and many new genes and signal pathways involved in this process have been identified. Some genes hold great promises as potential druggable targets. The genes that control EMT and cell motility as well as their signal pathways are rational candidates for the drug development. Although a clinical trial of the drugs that block MMP resulted in a rather disappointing outcome, these molecules are still considered to be excellent targets. The fact that metastatic cells are the only epithelial cells in circulation may provide us with a window of opportunity to attack such cells. In addition, tumor cells are often attracted by various types of chemokines to the distant organ sites, and these chemokines may also serve as molecular targets for anti metastatic therapy. Reactivation of metastasis suppressor genes and their signal pathways such as MKK/JNK, PTEN/Akt and NDRG/ATF are also a rational strategy. Recent finding that KAI1 blocks metastasis by inducing senescence upon interaction with endothelial cells also suggests an interesting possibility to develop an effective drug to activate the KAI1 pathway. Perhaps, genome wide shRNA library screening and comprehensive proteomics approach may reveal more suitable targets for metastatic therapy in the near future. The use of computer driven strategies such as automated determinations of the structures of target molecules and computer aided design of drug molecules followed by a high throughput screening has already begun to set this trend into motion.

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Fatty Acid Synthase Gene Is Up-regulated by Hypoxia via Activation of Akt and Sterol Regulatory Element Binding Protein-1

Eiji Furuta,[1] Sudha K. Pai,[1] Rui Zhan,[1] Sucharita Bandyopadhyay,[2] Misako Watabe,[1] Yin-Yuan Mo,[1] Shigeru Hirota,[3] Sadahiro Hosobe,[3] Taisei Tsukada,[3] Kunio Miura,[3] Shuichi Kamada,[3] Ken Saito,[3] Megumi Iiizumi,[1] Wen Liu,[1] Johan Ericsson,[4] and Kounosuke Watabe[1]

[1]Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine, Springfield, Illinois; [2]Department of Developmental Biology, Stanford University, School of Medicine, Stanford, California; [3]Akita Red Cross Hospital, Akita, Japan; and [4]Ludwig Institute for Cancer Research, Uppsala University, Biomedical Center, Uppsala, Sweden

Abstract

The fatty acid synthase (FAS) gene is significantly up regulated in various types of cancers, and blocking the FAS expression results in apoptosis of tumor cells. Therefore, FAS is considered to be an attractive target for anticancer therapy. However, the molecular mechanism by which the FAS gene is up regulated in tumor cells is poorly understood. We found that FAS was significantly up regulated by hypoxia, which was also accompanied by reactive oxygen species (ROS) generation in human breast cancer cell lines. The FAS expression was also activated by H_2O_2, whereas *N* acetyl L cystein, a ROS inhibitor, suppressed the expression. We also found that the hypoxia significantly up regulated sterol regulatory element binding protein (SREBP) 1, the major transcriptional regulator of the FAS gene, via phosphorylation of Akt followed by activation of hypoxia inducible factor 1 (HIF1). Moreover, our results of reporter assay and chromatin immunoprecipitation analysis indicate that SREBP 1 strongly bound to the SREBP binding site/E box sequence on the FAS promoter under hypoxia. In our xenograft mouse model, FAS was strongly expressed in the hypoxic regions of the tumor. In addition, our results of immunohistochemical analysis for human breast tumor speci mens indicate that the expressions of both FAS and SREBP 1 were colocalized with hypoxic regions in the tumors. Further more, we found that hypoxia induced chemoresistance to cyclo phosphamide was partially blocked by a combination of FAS inhibitor and cyclophosphamide. Taken together, our results indicate that FAS gene is up regulated by hypoxia via activation of the Akt and HIF1 followed by the induction of the SREBP 1 gene, and that hypoxia induced chemoresistance is partly due to the up regulation of FAS. [Cancer Res 2008;68(4):1003 11]

Introduction

Fatty acids have long chains of lipid carboxylic acid and play pivotal roles in normal cellular function as well as in homeostasis of the whole body. They are the source of membrane components, such as phospholipids and glycolipids, and also provide precursors of critical signal molecules for proliferation and differentiation (1). Fatty acids also function as a medium to store energy in the adipose tissue (2). In general, normal adult cells acquire fatty acid mainly from dietary source and rarely use the pathway of *de novo* synthesis, except in the liver, adipose tissue, and lactating mammary gland (3, 4). In striking contrast, many human tumor cells synthesize fatty acids by using the *de novo* pathway as was originally observed by Medes et al. (5) >50 years ago. Fatty acid synthase (FAS) is the major enzyme of lipogenesis and catalyzes the condensation of acetyl CoA and malonyl CoA to produce palmitic acid in the presence of NADPH (6). The FAS gene is highly up regulated in various types of human malignancies, although this gene is expressed at minimum or undetectable level in most normal tissues, and therefore, FAS overexpression is considered to be one of the most common molecular changes in cancer cells (7 11). Importantly, treatment of tumor cells with pharmacologic inhibitors of FAS leads to cell cycle arrest, followed by apoptosis of the tumor cells (12). We have previously shown that specific blocking of the FAS expression by using siRNA in breast cancer cells caused an accumulation of malonyl CoA, which led to the inhibition of carnitine palmitoyl transferase 1 as well as up regulation of ceramide (13). This was also followed by the induction of the proapoptotic genes, BNIP3, TRAIL, and DAP kinase 2, which resulted in the apoptosis of the tumor cells (13). These observations suggest that FAS overexpression confers selective advantage to tumor cells by inhibiting apoptosis and promoting cell cycle progression.

How the FAS gene is up regulated in cancer cells is an intriguing question, although it has been poorly understood. FAS was previously found to be up regulated by several growth factors and their receptors including epidermal growth factor, Her2 (ErbB2/neu), and keratinocyte growth factor (14 16). Upon binding to each receptor, these factors transmit cellular signals such as mitogen activated protein kinases (Erk1/2 MAPK2), Janus kinase (JNK), and phosphotidylinositol 3′ kinase (PI3K) followed by Akt activation (17 20). The activation of Akt is commonly observed in a variety of tumors and seems to contribute to the up regulation of the lipogenic enzymes. On the other hand, a lack of expression or mutation of the tumor suppressor gene, PTEN, has been well established in various types of tumors, and PTEN blocks the function of Akt by counteracting PI3K through dephosphorylation of this enzyme (21). In fact, we have recently shown that the expression of PTEN has a significant inverse correlation with FAS expression in prostate cancer patients (22), and that the inhi bition of the PTEN gene expression *in vitro* indeed led to the overexpression of FAS, although ectopic expression of PTEN significantly suppressed FAS (22). On the other hand, sterol regulatory element binding protein (SREBP) have been known to be the key transcription factors to regulate lipogenic genes, and the FAS gene was indeed shown to be significantly activated by SREBP 1. Interestingly, Porstmann et al. (20) recently found that Akt stimulated the synthesis and nuclear localization of activated

Requests for reprints: Kounosuke Watabe, Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, 801 North Rutledge Street, P.O. Box 19626, Springfield, IL 62794-9626. Phone: 217-545-3969; Fax: 217-545-3227; E-mail: kwatabe@siumed.edu.

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SREBP 1 followed by activation of the FAS gene. Therefore, PTEN Akt pathway and the downstream effectors play a critical role in the FAS gene regulation in cancer cells. Furthermore, FAS expression has also been found to be controlled by tumor suppressors and oncogenes, including p53, p63, p73, and H ras (18, 23). Therefore, overexpression of FAS is often associated with phenotypic changes of cell transformation that are induced either by oncogenes or tumor suppressors. These observations strongly suggest that FAS overexpression is actively contributing to the process of cell transformation rather than merely a consequence accompanied with the phenotypic changes.

Because FAS alone is not likely to cause cellular transformation but rather provides growth advantage to tumor cells by blocking proapoptotic genes, it is plausible that the FAS gene is up regulated by tumor microenvironment, such as hypoxia, a hallmark of tumors, as a survival strategy of tumor cells. This assumption is supported by the observation that the Akt pathway is activated by a hypoxic condition, and that Akt is also capable of activating SREBP 1, which is a key transcription factor of the FAS gene. In this article, we tested a possibility of regulation of FAS expression by hypoxia in breast tumor cells both *in vitro* and *in vivo* and found that the FAS gene is indeed up regulated by hypoxia through induction of Akt followed by activation of hypoxia inducible factor 1 (HIF1) and SREBP 1. We also found that hypoxia induced chemoresistance, which is a major clinical obstacle, can be partially overcome by a combination of a FAS inhibitor and a chemotherapeutic drug.

Materials and Methods

Cell culture and reagents. Human breast carcinoma cell lines, MX1, MCF7, MDA MB231, and MDA MB157 were purchased from American Type Culture Collection. The cells were maintained in RPMI 1640 supplemented with 10% FBS, streptomycin (100 μg/mL), penicillin (100 units/mL), and 250 nmol/L dexamethasone (Sigma Chemical Co.) and grown at 37°C in a 5% CO_2 atmosphere. The culture medium was replaced with DMEM before hypoxia treatment at 37°C in GasPak (BD Diagnostic Systems). Hydrogen peroxide, *N* acetyl L cysteine (NAC), cerulenin, YC 1, and cyclophosphamide were purchased from Sigma Chemical Co. LY294002 was obtained from Calbiochem. The expression plasmid of Akt1, Addgene plasmid 9008, which expressed activated (myristoylated) form of Akt1, was purchased from Addgene (24). siRNA for Akt and scramble sequence for control were obtained from Cell Signaling. siRNA for siSREBP was purchased from Santa Cruz Biotechnology.

Western blot. The cells were collected and resuspended in lysis buffer [50 mmol/L Tris Cl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, and 1 mmol/L EDTA]. The lysates were boiled for 5 min, resolved by SDS PAGE on an 8% polyacrylamide gel, and blotted onto nitrocellulose membrane. The membranes were treated with antibodies against FAS (0.2 μg/mL; Immuno biological Laboratories Co.), β tubulin (1:1,000; Upstate Biotechnology), HIF1 (1:200; BD Bioscience), SREBP 1 (1:200; Santa Cruz Biotechnology), phospho Akt (1:200; Ser^{473}; Cell Signaling), Akt (1:200; Cell Signaling), and phospho SREBP (0.5 μg/mL; ref. 25). The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies and visualized by ECL Plus system (Amersham Life Sciences).

Quantitative real time PCR. Total RNA was isolated from the cells and reverse transcribed. The cDNA was then amplified with a pair of for ward and reverse primers for the following genes: FAS (5′ CATCCAGA TAGGCCTCATAGAC 3′ and 5′ CTCCATGAAGTAGGAGTGGAAG 3′), SREBP (5′ CTGGTCTACCATAAGCTGCAC 3′ and 5′ GACTGGTCTTCACTCT CAATG 3′), and β actin (5′ TGAGACCTTCAACACCCCAGCCATG 3′ and 5′ CGTAGATGGGCACAGTGTGGGTG 3′). PCR reactions were performed using DNA engine opticon2 system (MJ Research) and the Dynamo SYBR Green qPCR kit (Finnzyme Corp.). The thermal cycling conditions composed of an initial denaturation step at 95°C for 5 min followed by 40 cycles of PCR using the following profile: 94°C, 30 s; 63°C, 30 s; and 72°C, 30 s.

Reactive oxygen species assay. The cells were cultured in RPMI medium and fluorophore dichlorodihydrofluorescein diacetate (DCFDA; Sigma Chemical Co.) was added directly to the medium at a final concentration of 50 μmol/L. The culture was further incubated at 37°C for 1 h, and the cells were washed with PBS. The stained cells were visualized under fluorescent microscope and photographed. The amount of staining was quantified by the MCID software.

Reporter assay. To generate the reporter plasmid for chloramphenicol acetyl transferase (CAT) reporter assay, the promoter region of FAS (from +4 to −1,328 bp) was amplified where the forward primer included the *Hin*d III linker and reverse primers included *Bgl* II linker. The PCR products were cloned into the pBLCAT3 plasmid. A series of deletions were constructed by Erase a Base System (Promega) according to the manufacturer's protocol. These plasmids were transfected to breast cancer cell line MCF7 by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h, the cells were collected and then subjected to CAT assay as described previously (26). The reaction was done, and acetylated [^{14}C]Chlor amphenicol was quantified with a PhosphorImager (Packard Instruments). The luciferase reporter plasmid of the FAS promoter was a gift from Dr. Verhoeven (Catholic University of Leuven, Leuven, Belgium; ref. 27). To delete eight bases of the SREBP binding/E Box sequence on the FAS promoter from this plasmid, QuickChange Site Directed Mutagenesis kit (Stratagene) was used according to the manufacturer's protocol. The luciferase reporter plasmids were transfected to MCF7 as described above. Luciferase activities were then measured by using Dual Luciferase Reporter Assay System (Promega) and Luminometer (Berthold Detection Systems). For each transformation experiment, the Renilla expression plasmid phRG TK (Promega) was cotransfected as an internal control, and promoter activities were normalized accordingly.

Chromatin immunoprecipitation. MCF7 cells were cultured in T75 flask and fixed with 1% formaldehyde for 10 min at room temperature. To stop the reaction, 125 mmol/L glycine was added to the culture medium, and the cells were washed with PBS and harvested. Cells were then suspended in cell lysis buffer (5 mmol/L PIPE, 85 mmol/L KCl, and 0.5% NP40) and homogenized with a type A Dounce homogenizer. The cell nuclei were collected and lysed with Nuclei lysis buffer (50 mmol/L Tris HCl, 10 mmol/L EDTA, and 1% SDS). The chromatin was sonicated on ice to an average length of 400 bp. The sample was then centrifuged at 4°C, and the precipitates were resuspended in chromatin immunoprecipitation (ChIP) dilution buffer (16.7 mmol/L Tris HCl, 167 mmol/L NaCl, 1.1% Triton X 100, and 0.01% SDS). After preclearing the sample with Protein G agarose beads (Dynal Biotech) followed by brief centrifugation, the supernatant was transferred to a new tube and anti SREBP 1 (Santa Cruz Biotechnology) antibody was added. After 24 h of incubation at 4°C, Protein G agarose beads were added, and the sample was incubated for 3 h at 4°C. The beads were then washed with washing buffer (100 mmol/L Tris HCl, 500 mmol/L LiCl, 1% NP40, and 1% deoxycholic acid), and DNA protein complexes were eluted with elution buffer (100 mmol/L $NaHCO_3$ and 1% SDS). DNA protein was decrosslinked followed by phenol extraction, and the purified DNA was subjected to PCR using both specific (5′ TCATTGGCCTGGGCGGCGCAG 3′ and 5′ AAACCGCGGCCATCCCCGGGC 3′) and nonspecific primers (5′ CAG CCAGAGACACCTGTGGCC 3′ and 5′ CCTTTTCTGACCGCTTCGCGC 3′) for the SREBP binding/E box sequence of the FAS promoter. The PCR products were visualized after electrophoresis on 8% acrylamide gel followed by staining with ethidium bromide.

Immunohistochemistry. Human breast cancer specimens were obtained from surgical pathology archives of the Akita Red Cross Hospital. All of the tissue sections were obtained by surgical resection. For immunohistochem ical staining, 4 μm thick sections were cut out from the formaldehyde fixed and paraffin embedded tissue specimens and mounted on charged glass slides. The sections were baked at 60°C for 1 h, deparaffinized by two changes of xylene, and rehydrated in graded alcohol solutions. For antigen retrieval, the sections were heated in 10 mmol/L sodium citrate (pH 6.0) at 85°C for 30 min. The slides were treated with 3% H_2O_2 to block endogenous peroxidase activity and then incubated overnight at 4°C with anti FAS rabbit polyclonal

antibody (0.2 μg/mL; Immuno biological Laboratories Co.), anti SREBP 1 rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology), or anti carbonic anhydrase 9 (CA9) mouse monoclonal antibody (1:100; R&D Systems, Inc.). The sections were then incubated with horseradish peroxidase conjugated anti rabbit or mouse IgG for 30 min at room temperature, and 3,3′ diaminobenzidine substrate chromogen solution [Envision plus kit (Dako Corp.) or ABC staining system (Santa Cruz Biotechnology)] was applied. Finally, the sections were counterstained with hematoxylin. Results of the immunohistochemistry were judged based on the intensity of staining, comparing the tumor cells and the normal glands on the same slide. Grading of the FAS, SREBP 1, and CA9 expression levels was done by two independent persons without prior knowledge of the patient data. The cases were then divided into those that showed positive staining and those that showed reduced expression of the two genes.

Animal model. Breast cancer cell line, MDA MB231, was suspended to 30 million/mL with PBS, and equal volume of Matrigel (BD Biosciences) was mixed with the tissue. The cell suspension (0.1 mL) was injected into the mammary fat pad of 4 week old female nude mice. A disc of 17B estradiol (Invitrogen) was also embedded under the skin of these mice. After 3 weeks, 0.2 mL of pimonidazole (Hypoxyprove 1 kit; Chemicon; 2,000 μg/mL) was injected to the mouse via i.p. After 2 h of the injection, the mouse was euthanized and the tumor excised and snap frozen. The tumor sample was cut into a 4 μm slice and mounted on charged glass slides. Immunohistochemical analyses were performed for these slices using anti pimonidazole and anti FAS antibodies.

***In situ* apoptosis assay.** The cells were grown in 96 well plates and fixed with 4% paraformaldehyde in PBS followed by permeabilization with 0.2% Triton X 100/0.1% sodium citrate at 4°C. The cells were then washed extensively and terminal deoxynucleotidyl transferase mediated dUTP biotin end labeling assay was performed using the *In Situ* Cell Death Detection kit/TMR Red (Roche Applied Science). The reaction was stopped after 1 h, and the number of apoptotic cells in each well was counted under a confocal microscope.

FAS enzyme assay. The enzyme activity of FAS was assayed as described previously (28). Briefly, the cells were grown in 12 well plates with or without cyclophosphamide and cerulenin. After 24 h, the cells were collected and resuspended with 0.25 mol/L Sucrose buffer (0.25 mol/L sucrose, 1 mmol/L EDTA, 5 mmol/L Tris HCl, and 1 mmol/L DTT), and the cells were then homogenized by a type A Dounce homogenizer. FAS activity was measured spectrophotometrically by monitoring oxidation of NADPH (Sigma). Fifty microliters of the cell extract were added to a 500 μL reaction mixture containing 0.1 mol/L K_2HPO_4 (pH 7.0), 0.3 mmol/L NADPH, and 0.05 mmol/L Acetyl CoA, and the absorbance at 340 nm was monitored for 3 min to measure background of NADPH oxidation. Malonyl CoA

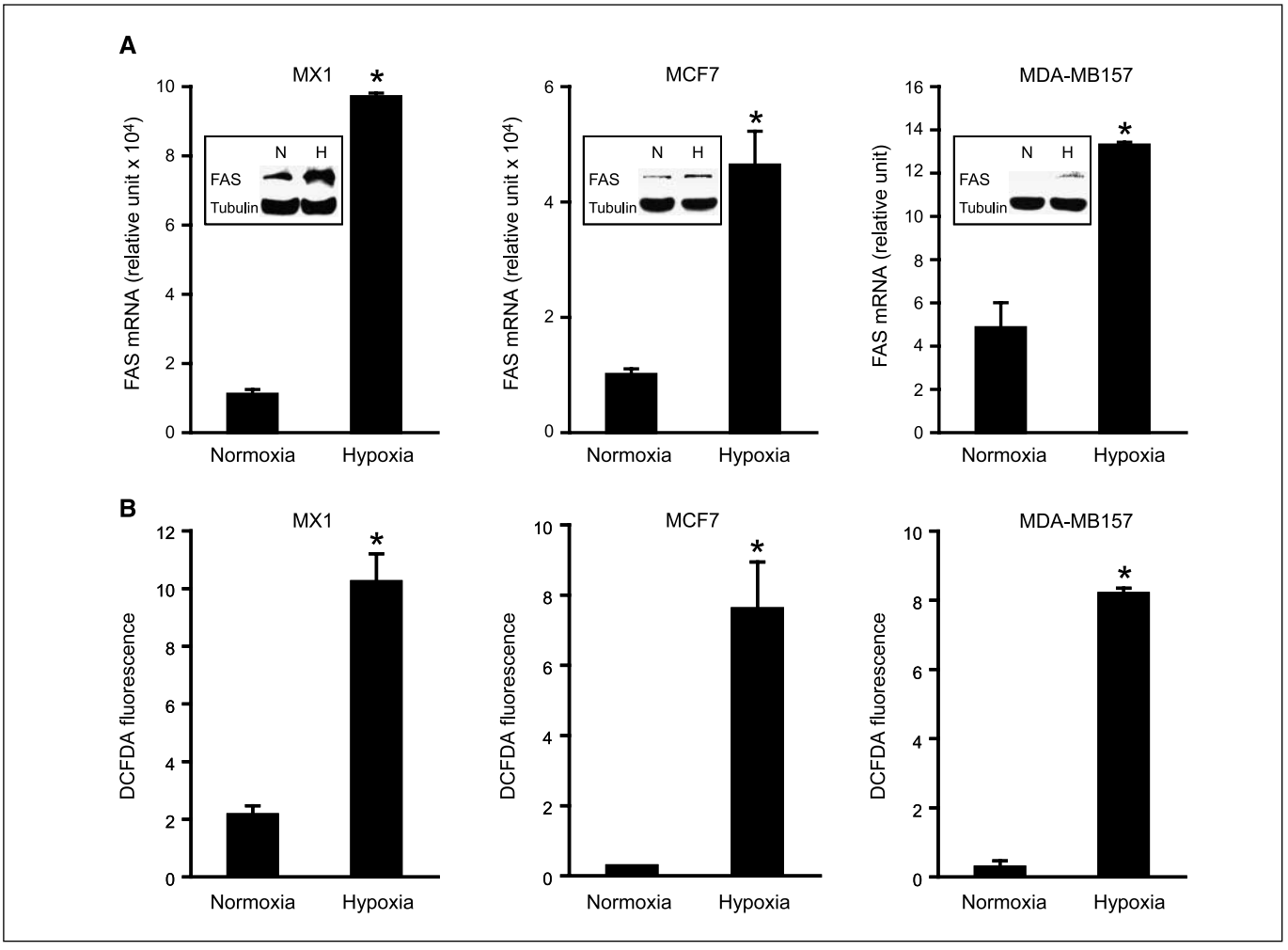


Figure 1. Hypoxia significantly augments the expression of FAS. *A,* human breast cancer cell lines, MX1, MCF7, and MDA MB157 were cultured in three sets of 24 well plates under normoxic (*N*) or hypoxic (*H*) conditions for 48 h. One set of cells (in triplicate) was collected, and RNA was prepared. The samples were then subjected to qRT PCR using primers for the FAS and β actin genes. Another set of cells was collected, and the cell lysates were subjected to Western blot analysis using anti FAS and antitubulin antibodies (*inset*). *B,* the last set of plates was used for assaying the amount of ROS using DCFDA dye. The cells were treated with the dye for 1 h followed by washing the wells with PBS. The stained cells were visualized under fluorescent microscope and photographed. The amount of staining was quantified by using a MCID software.

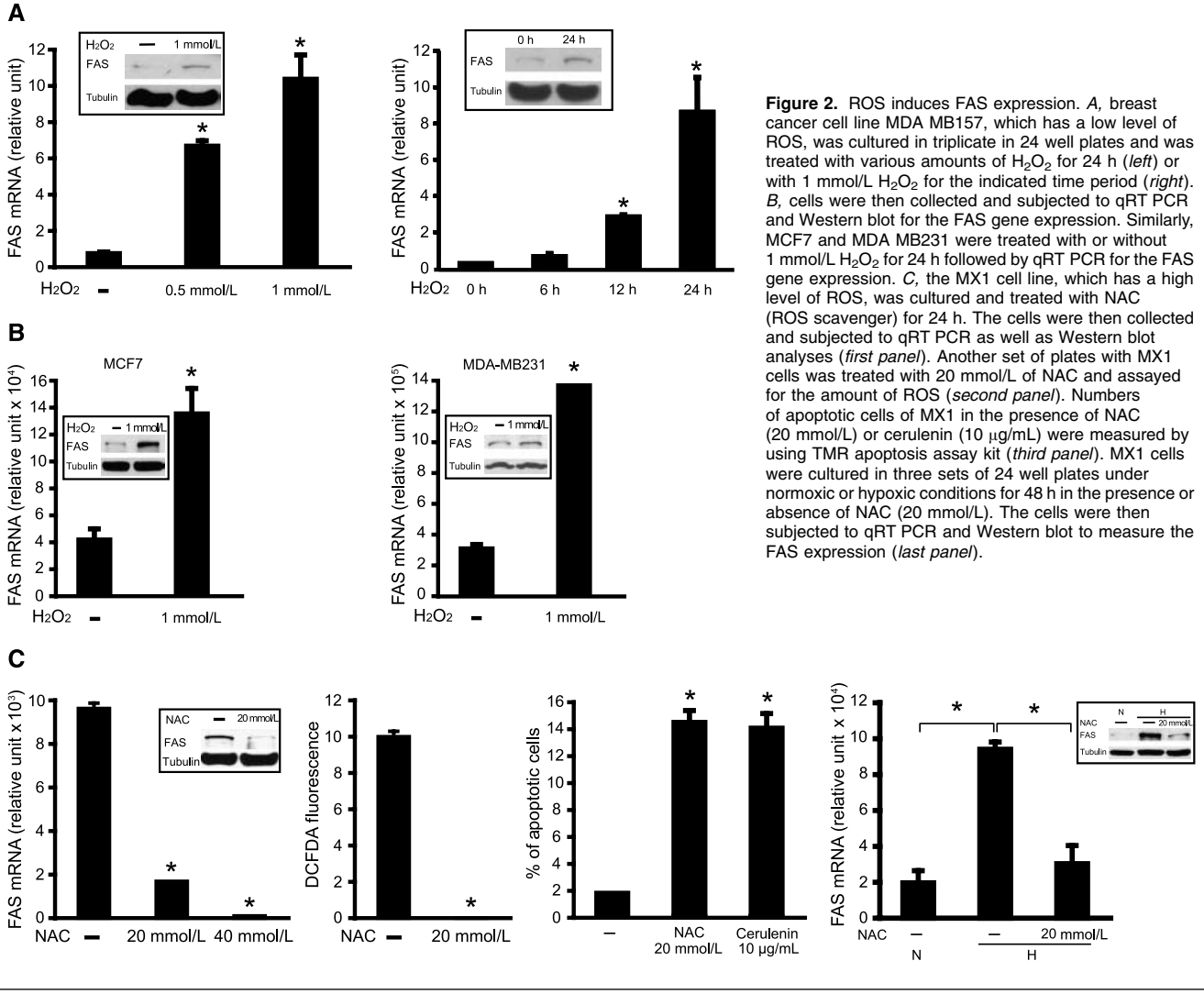


Figure 2. ROS induces FAS expression. *A,* breast cancer cell line MDA MB157, which has a low level of ROS, was cultured in triplicate in 24 well plates and was treated with various amounts of H_2O_2 for 24 h (*left*) or with 1 mmol/L H_2O_2 for the indicated time period (*right*). *B,* cells were then collected and subjected to qRT PCR and Western blot for the FAS gene expression. Similarly, MCF7 and MDA MB231 were treated with or without 1 mmol/L H_2O_2 for 24 h followed by qRT PCR for the FAS gene expression. *C,* the MX1 cell line, which has a high level of ROS, was cultured and treated with NAC (ROS scavenger) for 24 h. The cells were then collected and subjected to qRT PCR as well as Western blot analyses (*first panel*). Another set of plates with MX1 cells was treated with 20 mmol/L of NAC and assayed for the amount of ROS (*second panel*). Numbers of apoptotic cells of MX1 in the presence of NAC (20 mmol/L) or cerulenin (10 μg/mL) were measured by using TMR apoptosis assay kit (*third panel*). MX1 cells were cultured in three sets of 24 well plates under normoxic or hypoxic conditions for 48 h in the presence or absence of NAC (20 mmol/L). The cells were then subjected to qRT PCR and Western blot to measure the FAS expression (*last panel*).

(0.2 mmol/L) was then added to the reaction mixture, and absorbance at 340 nm was again monitored for 3 min to measure FAS activity.

Statistical analysis. For *in vitro* experiments, one way ANOVA was used to calculate the *P* values. Descriptive statistics comparing the expression of FAS, SREBP 1, and CA9 were analyzed by standard χ^2 test. For all of the statistical tests, the significance was defined as having a *P* value of <0.05. In all cases, SPSS software was used.

Results

Hypoxia induces the expression of the FAS gene via reactive oxygen species. To examine the effect of hypoxia on the expression of the FAS gene, we cultured three breast cancer cell lines, MX1, MCF7, and MDA MB157, under normoxic or hypoxic conditions. The RNA and cell lysates were prepared from these samples, and the level of FAS expression was measured by quantitative reverse transcription PCR (qRT PCR) and Western blot. As shown in Fig. 1, our results indicate that the transcription of the FAS gene was significantly increased in hypoxia compared with that in normoxia (Fig. 1*A*). Protein level of FAS was also strongly increased in hypoxic condition (Fig. 1*A, inset*), although the amount of FAS induced in these three cell lines at protein level (7.1 , 4.5 , and 3.0 fold, respectively) seems to be less than that at RNA level (8.7 , 4.6 , and 2.7 fold, respectively). These apparent differences may be due to the instability of the mRNA or the FAS protein. We also examined the amount of reactive oxygen species (ROS) in these cells under normoxic and hypoxic conditions and found that hypoxia significantly augmented the generation of ROS in all these cell lines (Fig. 1*B*), which is in good agreement with previous reports (29). These results suggest that the expression of the FAS gene is positively controlled by hypoxia, which is also associated with the amount of ROS in the cell. To further corroborate our results, we tested the effect of H_2O_2 on the FAS expression in MDA MB157, which displayed the lowest level of FAS. As shown in Fig. 2*A*, the addition of H_2O_2 in the culture medium significantly augmented the expression of FAS at both RNA and protein levels in a dose and time dependent manner. Other cell lines, MCF7 and MDA MB231, also showed a similar trend and increased the FAS expression by 3 to 4 fold in response to H_2O_2 (Fig. 2*B*). On the other hand, addition of an ROS scavenger, NAC, significantly suppressed the expression of the FAS gene as well as ROS production in MX1, which showed the highest level of FAS expression among the tested cell lines (Fig. 2*C, first* and *second*

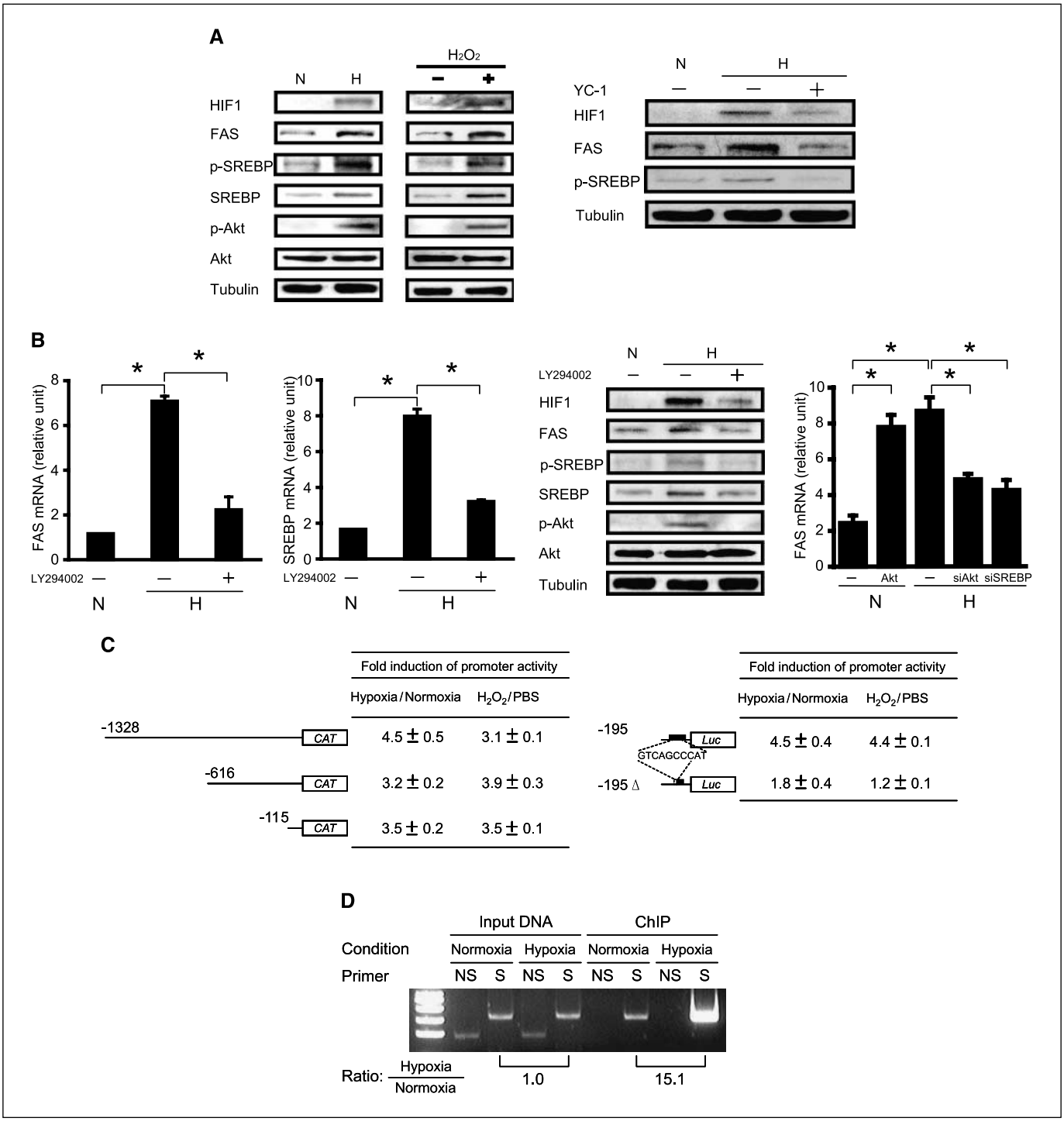
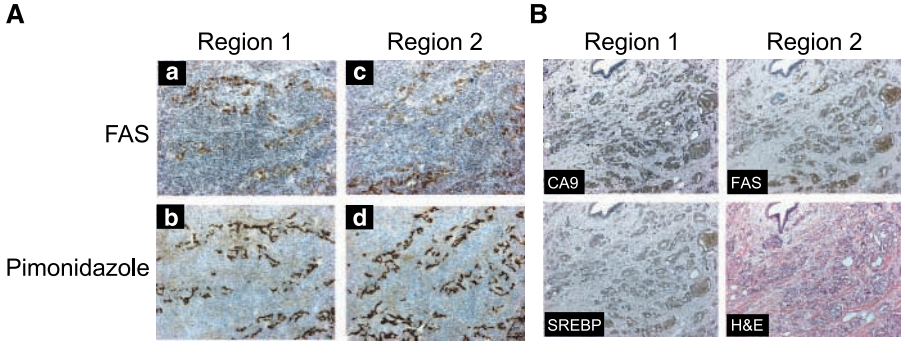


Figure 3. Hypoxia induced FAS expression is mediated via Akt, HIF1, and SREBP 1. *A,* MCF7 cells were cultured in 24 well plates under hypoxia or normoxia conditions for 48 h and with or without H_2O_2 for 24 h. Cells were then collected and subjected to Western blot analyses using antibodies for FAS, HIF1, phospho SREBP (*p SREBP*), SREBP 1, phospho Akt (*p Akt*), total Akt (*Akt*), and Tubulin (*left*). The MCF7 cells were also cultured in the presence or absence of YC 1 (HIF1 inhibitor) under hypoxic or normoxic conditions for 48 h. Cell lysates were subjected to Western blot analyses for HIF1 and FAS expression (*right*). *B,* MCF7 cells cultured under normoxia or hypoxia with or without the treatment of LY294002 were subjected to qRT PCR to quantify the expression of the FAS and SREBP genes (*first and second panels*). Another set of culture with the same treatment was also subjected to Western blot analysis (*third panel*). siRNA for Akt1 and SREBP 1, or the expression plasmid of active form of Akt1 were transfected to MCF7 cells. The cells were then incubated under normoxic or hypoxic conditions for 48 h. The cells were collected and subjected to qRT PCR analysis for FAS expression (*fourth panel*). *C,* CAT reporter constructs with various lengths of the FAS promoter were transfected to MCF7, and the cells were continued to be cultured under hypoxic or normoxic conditions for 48 h and with or without H_2O_2 for 24 h. Cells were then collected, and the cell lysates were subjected to CAT assay (*left*). The luciferase reporter plasmid with 195 bases of FAS promoter with or without deletion of E box was transfected to MCF7, and the cells were cultured under hypoxia or normoxia for 48 h and with or without H_2O_2 for 24 h. Cells were then collected and assayed for luciferase activities (*right*). *D,* for ChIP assay, MCF7 cells were cultured under normoxia or hypoxia for 24 h. The cells were lysed and the lysate was pulled down with anti SREBP 1 antibody. The DNA was then subjected to quantitative PCR using nonspecific (*NS*) or SREBP binding site specific primers (*S*). The ratio of the DNA was calculated based on cyclic threshold value for each reaction.

panels). Because inhibition of the FAS expression has been known to cause apoptosis (13), we also examined the effect of NAC on cell death. As shown in Fig. 2*C* (*third panel*), the treatment of the cell with NAC significantly induced apoptosis to the similar level as it was when treated with a specific inhibitor of FAS, cerulenin. To further confirm our results, we tested the effect of NAC on the FAS up regulation under hypoxia and found that NAC indeed significantly blocked the up regulation of FAS (Fig. 2*C, fourth panel*). Collectively, these results suggest that the expression of the FAS gene is up regulated by hypoxia through the generation of ROS.

Hypoxia up regulates the FAS gene expression through SREBP 1. To understand the mechanism of the hypoxia induced expression of the FAS gene, we first examined the status of HIF1, SREBP, and Akt under normoxic and hypoxic conditions. HIF1 has been known as a key transcriptional regulator induced by hypoxia (30). SREBP is the major transcription factor of the FAS gene and has been known to be up regulated under hypoxia (31). In fission yeast, SREBP was indeed found to function as an oxygen sensor (32). Akt is a key signal molecule for cell survival, and apoptosis and has been shown to be up regulated under hypoxia (33). As shown in Fig. 3*A* (*left*), our results of Western blot analysis indicate that expressions of FAS, HIF1, SREBP 1, and phospho SREBP (T426) were indeed up regulated under hypoxia as well as in the presence of H_2O_2 in MCF7 cells. We also found that Akt was strongly phosphorylated at Ser^{435} in the same set of samples treated with hypoxia or H_2O_2, although the amount of total Akt was somewhat decreased, suggesting that PI3K/Akt pathway and SREBP 1 are involved in the activation of FAS by hypoxia and ROS (Fig. 3*A, left*). Because HIF1 was also up regulated by hypoxia and H_2O_2, we next examined whether HIF1 was involved in the activation of FAS by adding a HIF1 inhibitor, YC 1, in the cultured cells under hypoxic condition. As shown in Fig. 3*A* (*right*), the hypoxic condition strongly up regulated HIF1, and this up regulation was blocked by YC 1. Interestingly, the YC 1 treatment also blocked the expression of FAS as well as phospho SREBP 1, suggesting that HIF1 is also involved in the up regulation of FAS and SREBP 1. The results of qRT PCR analysis also indicate that FAS and SREBP 1 were significantly increased by the treatment of hypoxia (Fig. 3*B, first* and *second panels*). Furthermore, the results of both of our qRT PCR and Western blot analyses indicate that the up regulation of FAS, p Akt, HIF1, and SREBP 1, as well as p SREBP, were blocked by LY294002 (Fig. 3*B, first, second*, and *third panels*), suggesting that the induction of the FAS expression by hypoxia is mediated through activation of Akt followed by up regulation of HIF1 and SREBP 1. To further verify our results, we tested the effect of siRNA specific to SREBP 1 and Akt as well as the effect of ectopic expression of an activated form of Akt on the FAS expression. We found that ectopic expression of Akt significantly augmented the FAS expression under normoxia, whereas both siRNA significantly blocked the up regulation of FAS under the hypoxic condition (Fig. 3*B, fourth panel*). Therefore, both Akt and SREBP 1 coordinately regulate the up regulation of hypoxia induced FAS expression.

To identify the exact hypoxia responding sequence on the FAS gene promoter, we generated a series of CAT reporter plasmids containing up to −1,328, −616, and −115 base of the FAS promoter, and CAT reporter activities were measured under normoxic or hypoxic conditions as well as in the presence or absence of H_2O_2. As shown in Fig. 3*C* (*left*), both hypoxia and H_2O_2 significantly increased the FAS promoter activity even when the promoter sequence was deleted to −115 bases. Because this region includes the SREBP binding/E box sequence, to assess the functional significance of these sequences, we generated luciferase reporter plasmids with or without the SREBP binding sequence and tested their responsive ness to hypoxia and H_2O_2. The results of the reporter assay indicate that deletion of the SREBP binding/E box sequence significantly reduced the responsiveness of the FAS promoter to hypoxia and H_2O_2 (Fig. 3*C, right*). Therefore, these results suggest that hypoxia induced the FAS gene by activating Akt followed by induction of SREBP 1, which then binds to the SREBP binding site of the FAS promoter. To examine further whether SREBP 1 indeed binds to the SREBP binding site under hypoxia, we performed ChIP assay by precipitating SREBP chromatin complex using anti SREBP 1



C

		CA9		
		Positive	Negative	*P* value *
FAS	Positive	21	9	*P* = 0.0038
	Negative	8	20	
SREBP	Positive	22	10	*P* = 0.0037
	Negative	7	19	

Figure 4. FAS and SREBP 1 express in hypoxic regions of tumor *in vivo*. *A,* MDA MB231 cells were transplanted into mammary fat pad of nude mice. The tumors were grown for 3 wk, and mice were injected with pimonidazole through i.p. After 2 h, tumors were excised and sliced on slides. These slides were then analyzed by immunohistochemistry using antibodies for FAS and pimonidazole. Photos are shown for two representative regions (*a b* and *c d*). *B,* to examine the relationship of FAS and hypoxia in tumor, human breast tumor samples from 29 patients were sectioned and subjected to immunohistochemical analysis using antibodies for FAS, SREBP 1, and CA9. Representative photos for each antibody staining with consecutively sectioned slides are shown. *C,* for each slide, fields of high and low expression of CA9 were randomly chosen and divided into two groups (CA9 positive and CA9 negative). Using consecutive slides of the identical samples, these regions were then analyzed by immunohistochemistry using anti FAS and anti SREBP 1 antibodies. Each sample was then further divided according to positive and negative expression of FAS and SREBP 1. To evaluate the significance, the expression of FAS and SREBP 1 in relation to CA9, χ^2 test was performed.

antibody followed by PCR, using the primers specific to the region of the SREBP binding site. Our result clearly indicates that the amount of SREBP 1 binding to this region was strongly augmented under hypoxia compared with that in normoxic condition (Fig. 3*D*).

The level of FAS expression correlates with hypoxia *in vivo*. To validate our *in vitro* results of the hypoxia induced FAS expression, we examined the relationship between the expression level of FAS and hypoxic regions in an animal xenograft model. We first transplanted human breast cell lines, MDA MB231, into nude mice and grew the tumor for 3 weeks. We then injected pimonidazole, which reacts with hypoxic cells, to the mice through i.p. After 2 h, tumors were excised and stained with antibodies for FAS and pimonidazole. We found that FAS expression colocalized with the area reactive to anti pimonidazole, suggesting that hypoxic areas strongly expressed FAS in these tumors (Fig. 4*A*). To further validate these results in a clinical setting, we performed immunohistochemical analysis for clinical samples from 29 breast cancer patients using antibodies for FAS, SREBP 1, and CA9, which is a hypoxia marker (34). We first stained the samples with anti CA9 and randomly chose positive and negative fields for each specimen. These samples were then stained with antibodies for FAS and SREBP 1. We then inspected the staining intensity of FAS and SREBP 1 in these CA9 positive and CA9 negative regions. As shown in Fig. 4*B* and *C*, of 29 CA9 positive regions, 21 were FAS positive (72%) and 8 were FAS negative. On the other hand, 20 of 29 CA9 negative samples (69%) were also FAS negative ($P = 0.0038$). CA9 positive regions were also significantly correlated with SREBP 1 expression ($P = 0.0037$). These results indicate that FAS was expressed preferentially in the region of hypoxia in breast cancer, which is consistent with our *in vitro* data. Taken together, our results of *in vitro* and *in vivo* experiments strongly suggest that the expression of the FAS gene is significantly induced by hypoxia, and that this induction is mediated by the generation of ROS followed by the activation of Akt and SREBP 1.

Inhibition of FAS overcomes hypoxia induced chemoresist ance. Development of resistance to chemotherapeutic drugs is a major clinical problem for the treatment of cancer patients. Rapidly growing tumors are often under hypoxic conditions, and hypoxia is known to induce chemoresistance (33, 35, 36). Because our results suggest that FAS is induced by hypoxia and that the high level of FAS protects tumor cells from apoptosis, we sought a possibility that inhibition of FAS expression by low concentra tion of a FAS inhibitor overcomes the hypoxia induced chemo resistance. We first examined the effect of cyclophosphamide, a chemotherapeutic drug commonly used for the treatment of breast cancer, on MCF7 cells under hypoxic or normoxic condition. As shown in Fig. 5*A*, cyclophosphamide induced apoptosis in MCF7 in a dose dependent manner under normoxic condition. However, when the cells were treated with cyclophosphamide under hypoxic condition, cells became significantly resistant to cyclophosphamide, indicating that hypoxia induced chemoresistance. We then treated MCF7 cell with a combination of a FAS inhibitor (cerulenin), a PI3K inhibitor (LY294002), and cyclophosphamide under hypoxic or normoxic condition followed by apoptosis assay. We found that a combination of these drugs synergistically enhanced the degree of apoptosis under normoxic condition (Fig. 5*B*). Importantly, the treatment of the cells with the combination of cyclophosphamide and cerulenin or LY294002 under hypoxia condition blocked hypoxia induced resistance to cyclophosphamide. These results suggest that a combination of cerulenin and other chemothera peutic drugs such as LY294002 synergistically induces tumor cell

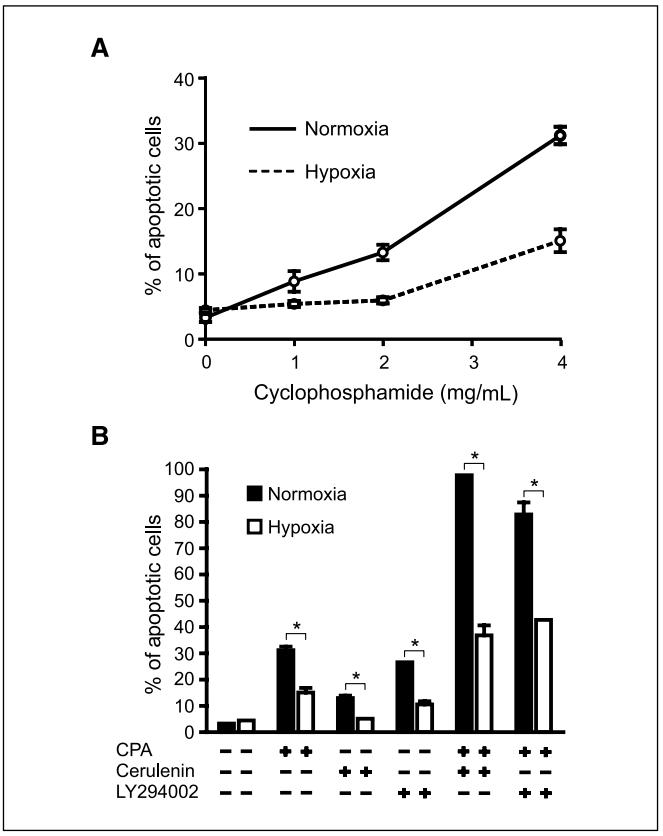


Figure 5. Inhibition of FAS overcomes hypoxia induced chemoresistance. *A,* MCF7 cells were treated with various amounts of cyclophosphamide under normoxic (*solid line*) or hypoxic (*dotted line*) conditions for 48 h. Cells were then subjected to apoptosis assay using the TMR apoptosis assay kit. *B,* MCF7 cells were treated with suboptimum concentrations of cerulenin (10 μg/mL), LY294002 (20 μmol/L), and cyclophosphamide (4 mg/mL) either alone or in combination under hypoxia or normoxia for 48 h. Samples were then subjected to apoptosis assay as described in *A. CPA,* cyclophosphamide.

death, and that hypoxia induced chemoresistance is partially blocked by suppression of the FAS expression or the Akt pathway. We also examined the enzymatic activity of FAS and found that the FAS activity was indeed significantly higher under hypoxic con dition compared with that under normoxia even in the presence of cyclophosphamide and cerulenin (3.8 ± 1.1 versus 1.1 ± 0.2, respectively), and that the activity was inversely correlated with the degree of apoptosis. Although currently available FAS inhibitors are relatively toxic, using these drugs at a low concentration with a combination of other drugs may be a rational strategy for the treatment of chemoresistant tumors.

Discussion

Although the *de novo* pathway of fatty acid synthesis is quite active during embryogenesis, normal adult cells acquire fatty acids mainly from dietary source and rarely use the *de novo* pathway because nutritional fatty acid strongly suppresses the expression of the genes involved in fatty acid synthesis (3, 4). However, cancer cells are no longer sensitive to this nutritional signal and prefer to use the *de novo* pathway. In fact, linoleic and arachidonic acid, potent suppressors of the FAS gene of normal hepatic and adipocytic cells, have been shown to have no significant inhibitory effect on the expression of the FAS gene in breast cancer cells (37). Therefore, what triggers the reactivation of the FAS gene in cancer

cells and whether they use the same signal pathway as the normal cells are critical questions to understand the role of FAS in tumorigenesis. When primary tumor grows >1 mm in size, it can no longer obtain oxygen and nutrients by diffusion and requires to promote angiogenesis by inducing proangiogenic genes as a survival strategy (36). Therefore, tumor cells at an early stage are usually under hypoxic condition and at a risk of apoptosis. The reactivation of the FAS gene has been observed at a relatively early stage in various types of cancer, and these results suggest that the FAS gene is up regulated by a common factor of cancer microenvironment such as hypoxia. In this report, we have shown that the FAS gene in cancer cell is indeed significantly up regulated by hypoxia, and that this up regulation is due to the activation of the Akt and HIF1 followed by up regulation of SREBP 1.

Due to the high rate of proliferation and oxygen consumption, tumors are often under hypoxic condition, which is a hallmark of cancer. The hypoxic microenvironment is normally proapoptotic; however, tumor cells adapt themselves by inducing various enzymes to circumvent the problem. This induction is mediated by an activation of the known hypoxia sensing pathways such as HIF1 and PI3K/Akt (38 40). In this context, it should be noted that Akt has been shown to stabilize HIF1 in both breast and prostate cancer cells (41, 42). Beitner Johnson et al. (43) also showed that hypoxia dramatically increased phospho Akt (Ser^{473}) in PC3 cells, and this activation of Akt was completely abolished by wortmannin, a PI3K inhibitor. It is worth noting that Akt was also found to be up regulated by H_2O_2 (44). Consistent with these results, we have shown that hypoxia and H_2O_2 indeed induced activation of Akt (Ser^{473}) and HIF1, and that this activation was accompanied by the up regulation of SREBP 1, a major factor involved in the regulation of the FAS gene. In cancer cells, it has been shown that PI3K/Akt signaling significantly augmented the expression of SREBP 1 in response to oncogenic signaling, including overexpression of various growth factors (11). Furthermore, we have previously shown that the tumor suppressor, PTEN, which inhibits Akt by dephosphorylation, significantly suppressed the expression of the FAS gene (22). Therefore, the activation of the Akt pathway followed by induction of SREBP 1 is considered to be one of the major pathways of reactivation of the FAS gene in cancer cell, and this reactivation is triggered at least by the hypoxic condition of tumor microenviron ment. This notion is also strongly supported by our results of immunohistochemical analysis on clinical samples where FAS expression was significantly colocalized with the CA9 stained hypoxic area. It is known that Akt is quickly phosphorylated under hypoxic condition and that this activation of Akt results in up regulation of HIF1 (45 47). Our results indeed showed that LY294002 inhibited hypoxia induced HIF1 as well as the expression of FAS and p SERBP 1 (Fig. 3*B*). Our results also indicate that HIF1 inhibitor, YC 1, strongly blocked phosphorylation of SREBP 1 (Fig. 3*A*), which is in good agreement with the recent finding by Li et al. (48) that HIF1 plays a key role in activation of SREBP 1 *in vivo*. Therefore, the hypoxia induced FAS expression is considered to be mediated via phosphorylation of Akt followed by activation of HIF1 and SREBP 1.

Hypoxia generally induces apoptosis in normal cells partly due to malfunction of the respiratory system in mitochondria, which requires oxygen for ATP production (49). However, cancer cells have an unusual tolerance to hypoxic condition because they use the glycolysis pathway to generate ATP even under normoxic condition, which has been known as the Warburg effect (50). On the other hand, hypoxia was shown to cause an increase of NADH/NADPH ratio in a cell due to increased flux of glycolysis, and this change of redox balance induces inactivation of PTEN followed by activation of Akt (21). Therefore, up regulation of FAS may be partly due to increased glycolysis and the following Akt activation. It is likely that the increased activity of FAS enhances lipogenesis, which consumes more NADPH and rebalances redox so that cells can compensate for the shortfall of oxygen.

As we and others previously reported, inhibition of the function or expression of FAS results in apoptosis of tumor cells (7 11). This cell death is considered to be caused by the suppression of CPT1 followed by accumulation of ceramide, which in turn activates proapoptotic genes such as BNIP3 (13). It should be noted that BNIP3 was found to be one of proapoptotic genes induced by hypoxia, and that specific blocking of the FAS expression by siRNA significantly increased the expression of BNIP3 followed by apoptosis (13, 51). In fact, we have shown that the expressions of FAS and BNIP3 are indeed inversely correlated in breast cancer patients (13). Therefore, FAS may act as an "antiapoptotic" gene under hypoxia. This notion is consistent with the previous observations of immunohistochemical analysis on human tumor samples where overexpression of FAS was found to be a relatively early event (7 11). We also reported that the expression of FAS was inversely related to that of PTEN in human breast tumor specimens, and the expression of higher FAS and lower PTEN is correlated to poor survival of patients, suggesting that the PTEN inactivation followed by Akt activation induced the FAS expression (22). Although the direct involvement of FAS in the initial step of tumorigenesis is yet to be determined, overexpression of FAS in tumors seems to be a survival strategy of the cancer cells to block apoptosis caused by hypoxic condition.

Because inhibition of FAS causes tumor cell apoptosis, FAS is considered to be a promising target for cancer therapy. The pharmacologic inhibitors of FAS such as cerulenin [(2*R*, 3*S*) 2,3 epoxy 4 oxo 7, 10 *trans,trans* dodecadienamide], C75, and Orlistat have been shown to significantly suppress the cellular FAS level and also to induce apoptosis in a variety of human cancer cells including breast, prostate, colon, and ovarian cancer, although their specificity of action and potential side effects remains to be of some concern for actual clinical use (7 11). On the other hand, traditional chemotherapeutic agents commonly used for breast cancer treatment such as cyclophosphamide, carboplatin, and doxorubicin often become ineffective due to chemoresistance, particularly under hypoxic condition (52). The exact mechanism of the hypoxia induced chemoresistance has not been well under stood; however, one possible mechanism is the activation of the Akt pathway and following expression of antiapoptotic genes including FAS (33). Our results of the *in vitro* experiments clearly indicate that a FAS inhibitor, cerulenin, indeed partially over came the hypoxia induced chemoresistance of cyclophosphamide. Although the existing FAS inhibitors are still somewhat toxic, a use of lower concentration of these drugs in combination with the current chemotherapeutic drugs may enhance the therapeutic effect by reducing the hypoxia induced chemoresistance.

Acknowledgments

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RhoC Promotes Metastasis via Activation of the Pyk2 Pathway in Prostate Cancer

Megumi Iizumi,[1] Sucharita Bandyopadhyay,[2] Sudha K. Pai,[1] Misako Watabe,[1] Shigeru Hirota,[3] Sadahiro Hosobe,[3] Taisei Tsukada,[3] Kunio Miura,[3] Ken Saito,[3] Eiji Furuta,[1] Wen Liu,[1] Fei Xing,[1] Hiroshi Okuda,[1] Aya Kobayashi,[1] and Kounosuke Watabe[1]

[1]Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine, Springfield, Illinois; [2]Department of Developmental Biology, Stanford University School of Medicine, Stanford, California; and [3]Akita Red Cross Hospital, Akita City, Japan

Abstract

***RhoC* is a member of the Ras homologous family of genes which have been implicated in tumorigenesis and tumor progression. However, the exact role of RhoC is controversial and is yet to be clarified. We have examined the effect of RhoC on prostate tumor cells and found that RhoC had no effect on cell proliferation *in vitro* or on tumor growth in mice. However, RhoC significantly enhanced the metastatic ability of the tumor cells in these animals, suggesting that RhoC affects only the metastasis but not the growth of prostate tumor cells. The results of our immunohistochemical analyses on tumor specimens from 63 patients with prostate cancer indicate that RhoC expression had no significant correlation with Gleason grade. However, the expression of RhoC showed significant positive correlation with both lymph node and distant metastasis, and it was inversely correlated with patient survival. We also found that RhoC significantly augmented the invasion and motility of prostate tumor cells by activating matrix metalloproteinases 2 and 9 (MMP2 and MMP9) *in vitro*. The results of our antibody array analysis for signal molecules revealed that RhoC significantly activated kinases including mitogen activated protein kinase (MAPK), focal adhesion kinase (FAK), Akt, and Pyk2. Inhibition of Pyk2 kinase blocked the RhoC dependent activation of FAK, MAPK, and Akt, followed by the suppression of MMP2 and MMP9. Inhibitors of both MAPK and Akt also significantly blocked the activities of these MMPs. Therefore, our results indicate that RhoC promotes tumor metastasis in prostate cancer by sequential activation of Pyk2, FAK, MAPK, and Akt followed by the up regulation of MMP2 and MMP9, which results in the stimulation of invasiveness of tumor cells.** [Cancer Res 2008;68(18):7613–20]

Introduction

The family of Ras homologous (*Rho*) genes, which plays a central role in cell proliferation and motility, has been implicated in tumorigenesis as well as metastatic progression (1). The Rho subfamily includes RhoA, RhoB, and RhoC and they share 85% amino acid sequence identity (2). Despite this similarity, each protein has different affinities with various downstream effectors and shows different subcellular localizations, suggesting that they have distinct roles in normal cellular function as well as in tumor pathogenesis (3). RhoA seems to be involved in the regulation of actomyosin contractility, and the overexpression of RhoA has been shown to promote the invasiveness of tumor cells (2, 4–6). On the other hand, RhoB plays a role in controlling cytokine trafficking as well as in apoptosis induced by DNA damaging agents and has been suggested to act as a suppressor of tumor progression (7, 8).

Recently, RhoC has been shown to be up regulated in various types of cancer including inflammatory breast cancer (9), hepatocellular carcinoma (10), and non small cell lung cancer (11). However, the exact role of RhoC in tumorigenesis and tumor progression has remained controversial and needs further clarification. Pillé and colleagues previously found that blocking *RhoC* expression by short interfering RNA significantly inhibited cell proliferation of breast tumor cells *in vitro* as well as tumor growth in an animal model (12). More recently, Faried and colleagues also reported that ectopic expression of RhoC in esophageal carcinoma cells significantly enhanced the growth of tumors in nude mice. These results suggest that RhoC plays a critical role in cell proliferation and tumor growth both *in vitro* and *in vivo* (13). On the contrary, Ikoma and colleagues reported that ectopic expression of RhoC using retroviral vectors in Lewis lung carcinoma cells showed no significant difference in primary tumor growth in mice. However, the rate of lymph node metastasis was significantly enhanced in these animals (14). In agreement with these results, Hakem and colleagues recently constructed a RhoC knockout mouse and found that loss of RhoC does not affect tumorigenesis but significantly decreased metastasis in this mouse, suggesting that RhoC is involved only in metastasis but not in tumor cell proliferation (15). These apparent contradictory results by different groups may be due to the difference in the systems used or it may be due to the dependency of RhoC on cellular context. Therefore, it is critical to take a more systematic approach of testing the gene both *in vitro* and *in vivo* and to validate the outcome results in a clinical setting for each organ or tissue type in order to further clarify the role of RhoC in tumor progression. In this study, we found that RhoC promotes tumor metastasis but not tumor growth by sequential activation of Pyk2, focal adhesion kinase (FAK), mitogen activated protein kinase (MAPK), and Akt followed by up regulation of matrix metalloproteinases 2 and 9 (MMP2 and MMP9) in prostate tumor cells, and that the expression of RhoC serves as a marker to predict metastatic status and survival of patients with prostate cancer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Kounosuke Watabe, Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, 825 North Rutledge Street, Springfield, IL 62702. Phone: 217-545-3969; Fax: 217-545-3227; E-mail: kwatabe@siumed.edu.

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Materials and Methods

Cell culture and reagents. Human prostate cancer cell line PC3 was obtained from American Type Culture Collection, and human prostate cancer cell line PC3MM was kindly provided by Dr. I.J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The PC3MM/tet cell line was previously established as a derivative of PC3MM and contains the tetracycline inducible suppressor. Rat prostate cancer cell line AT2.1 was a gift from Dr. C.W. Rinker Schaeffer (University of Chicago, Chicago, IL). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL), penicillin (100 units/mL), and 250 nmol/L of dexamethasone at 37°C in a 5% CO_2 atmosphere. The phosphoinositide 3 kinase (PI3K)/Akt inhibitor (Ly294002) and the MAPK inhibitor (PD98059) were purchased from Sigma Co. and Calbiochem, respectively. FAK inhibitor (TAE226) was previously described and kindly provided by Dr. Honda (Novartis Pharma AG, Basel, Switzerland; ref. 16).

Construction of expression vectors. To generate a RhoC expression vector, cDNA of the RhoC gene was isolated by PCR amplification from a human cDNA library using a forward primer containing a Flag tagged Kozak sequence and *Eco*RI linker and a reverse primer including a *Xho*I linker. The PCR product was then cloned into the mammalian expression vector pcDNA3 (Invitrogen). To construct a tetracycline inducible RhoC expression plasmid, the fragment of the *RhoC* gene in pcDNA3 was subcloned into pcDNA5/TO (Invitrogen) at the *Bam*HI/*Xho*I site. The RhoC expression plasmids or the vector alone were transfected into the AT2.1, PC3MM, and PC3MM/tet cells using LipofectAMINE (Invitrogen). To establish stable clones, transfected cells were treated with G418 or hygromycin, and drug resistant colonies were selected followed by testing RhoC expression by Western blot.

Short hairpin RNA. Five individual short hairpin RNAs (shRNA) against the *Pyk2* gene were purchased from Open Biosystems. shRNA with a scrambled sequence was purchased from Addgene and used as a negative control. The shRNAs were transfected into the prostate cancer cells using LipofectAMINE (Invitrogen) according to the manufacturer's protocol, and the culture was further incubated for 48 h before harvesting the cells for assays.

Western blot analysis. Cells were collected and dissolved in loading dye solution (125 mmol/L Tris HCl, 4% SDS, 20% glycerol, 10% β2 mercaptoethanol, and 0.04% bromophenol blue), boiled for 5 min and subjected to 8% to 12% SDS PAGE. Proteins were transferred to nitrocellulose membranes that were then treated with antibodies against anti Flag (Sigma Aldrich), anti β tubulin (Upstate Biotechnology), anti phospho Pyk2 ($Tyr^{579/580}$; Sigma Aldrich), anti Pyk2 (Cell Signaling Technology), anti phospho Akt (Ser^{473}; Cell Signaling Technology), anti Akt (Cell Signaling Technology), anti phospho FAK (Tyr^{397}; Sigma Aldrich), anti FAK (Cell Signaling Technology), or anti phospho MAPK (Thr^{183}; Sigma Aldrich) or anti MAPK (Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies and visualized by the enhanced chemiluminescence plus system (Amersham Life Sciences).

Cell growth assay. Cell lines expressing or not expressing the *RhoC* gene were cultured in the RPMI 1650 medium. At each time point, cells were trypsinized, serially diluted, and re plated in Petri dishes. The resultant colonies were stained with crystal violet and the number of colonies was visually counted. For thymidine uptake assays, cells were treated with or without tetracycline for 24 h and ^3H thymidine was added to the culture. After 3 and 12 h, cells were collected and acid insoluble radioactivities were measured by scintillation counter.

Spontaneous metastasis assay. Rat prostate tumor cells AT2.1 (0.5×10^6 cells in 0.2 mL of PBS) were injected s.c. in the dorsal flank of 5 week old severe combined immunodeficiency (SCID) mice (Harlan Sprague Dawley). Mice were monitored daily and the tumor volume was measured as an index of the growth rate using the equation: volume = (width + length) / 2 × width × length × 0.5236. The doubling time of tumors during the fastest growing period was calculated by measuring the tumor volume every 4 days. Mice were sacrificed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically.

Immunohistochemical analysis. Formaldehyde fixed and paraffin embedded tissue specimens from 63 patients with prostate cancer were obtained from surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan). Four micron thick sections were cut from the paraffin blocks of prostate tumors and mounted on charged glass slides. The sections were deparaffinized and rehydrated, and antigen retrieval was done by heating the slide in 25 mmol/L of sodium citrate buffer (pH 9.0) at 80°C for 30 min. The slides were incubated overnight at 4°C with anti RhoC antibody (Santa Cruz Biotechnology) or anti phospho Akt (Ser^{473}; Cell Signaling Techno logy). The sections were then incubated with the horseradish peroxidase conjugated anti goat secondary antibody, and 3,3′ diaminobenzidine substrate chromogen solution (Envision Plus kit; DAKO, Corp.) was applied followed by counterstaining with hematoxylin. Immunohistochemical staining conditions with other antibodies (NDRG1, AR, and PTEN) were described previously (17). Results of the immunohistochemistry for RhoC were judged by two independent persons (M. Iiizumi and K. Watabe) based on the intensity of staining combined with the percentage of cells with positive staining.

***In vitro* motility and invasion assay.** For the motility assay, 1×10^5 cells were added to the cell culture inserts with microporous membrane without any extracellular matrix coating (Becton Dickinson) and RPMI medium containing 20% fetal bovine serum was added to the bottom chamber. The cells were then incubated for 24 h at 37°C, and the upper chamber was removed. The cells on the bottom of the upper chambers were stained with tetrazolium dye, and the number of cells was counted under a microscope. For the *in vitro* invasion assay, the working method was similar to that described above, except that the inserts of the chambers to which the cells were seeded were coated with Matrigel (Becton Dickinson).

Wound healing migration assay. Cells were seeded in a 10 cm dish and cultured to confluency. The cell monolayer was then scraped in the form of a cross with a plastic pipette tip. Three "wounded" areas were marked for orientation and photographed by a phase contrast microscopy before and after 24 h of incubation.

Real time reverse transcription PCR. Forty eight hours after transfec tion of appropriate plasmid DNA to the cells or 48 h after induction by tetracycline, total RNA was isolated from the cells and reverse transcribed using random hexamer and MuLV reverse transcriptase (Applied Bio systems). The cDNA was then amplified with a pair of forward and reverse primers for *RhoC* (5′ TAAGAAGGACCTGAGGCAAG and 5′ ATCTCAGA GAATGGGACAGC), *MMP2* (5′ TGATGGTGTCTGCTGGAAAG and GACACGTGAAAAGTGCCTTG), *MMP9* (5′ GGAGACCTGAGAACCAATCTC and 5′ TCCAATAGGTGATGTTGTGGT), human *β actin* (5′ TGAGACCTT CAACACCCCAGCCATG and 5′ GTAGATGGGCACAGTGTGGGTG), *Pyk2* (5′ GCTAGACGGCAGATGAAAGT and 5′ AAGCAGACCTTGAGGATACG). PCRs were done using the Dynamo SYBRGreen qPCR kit (New England Biolabs) and DNA Engine Opticon2 System (MJ Research). The thermal cycling conditions were composed of an initial denaturation step at 95°C for 5 min followed by 30 cycles of PCR using the following profile: 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s.

Gelatin zymograph assay. For zymography assay, cells (2.5×10^5) were seeded in 12 well plates and incubated for 48 h. Supernatants were collected and mixed with sample buffer followed by electrophoresis on a 10% SDS polyacrylamide gel containing 5 mg/mL of gelatin. The gel was washed with 2.5% Triton X solution for 2 h and further incubated in the reaction buffer (50 mmol/L Tris HCl, 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, and 1% Triton X 100) for an additional 18 h at room temperature. The gel was then stained with 0.5% Coomassie blue for 9 h and subsequently immersed with destaining buffer (30% methanol, 10% acetic acid) for 12 h. The image was photographed and the intensity of each band was digitally quantified.

Antibody microarray. Antibody microarray was performed using a Panorama Antibody Microarray Cell Signaling kit (Sigma Aldrich) according to the manufacturer's instructions. Briefly, 1.5×10^7 cells were seeded in T 75 flasks and incubated for 48 h in the medium with or without tetracycline. Cells were collected and protein samples were prepared according to the manufacturer's protocol. These protein samples were labeled with Cy3 or Cy5 (Amersham Biosciences, UK) and subjected to antibody microarray (Sigma Aldrich) analysis. The array slides were

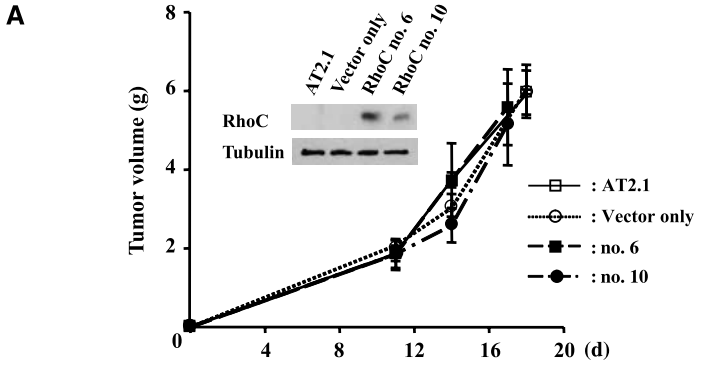
scanned by GenePix Personal 4100A scanner (Molecular Devices) and the data was analyzed by GenePix Pro 5.0 (Molecular Devices).

Statistical analysis. For *in vitro* experiments and animal studies, *t* test or one way ANOVA was used to calculate the *P* values. The association between RhoC and other clinical markers was calculated by χ^2 test. The Kaplan Meier method was used to calculate the overall survival rate, and prognostic significance was evaluated by the log rank test. Univariate and multivariate analyses for the prognostic value of RhoC was performed by the Cox proportional hazard regression model. For all of the statistical tests, the significance was defined as $P < 0.05$. SPSS software was used in all cases.

Results

RhoC promotes tumor metastasis, but not cell growth. To understand the role of RhoC in prostate cancer, we first established permanent cell lines expressing RhoC using the rat prostate carcinoma cell line, AT2.1, which has a poor metastatic potential (18). These cell lines expressing RhoC (clone no. 6 and no. 10) and a clone containing only the vector as well as the parental cell line, AT2.1, were individually injected s.c. into SCID mice. The mice were monitored for the formation and the growth rate of tumors and then sacrificed 3 weeks after the inoculation of the cells. As shown in Fig. 1*A*, all of the clones and the parental cells formed primary tumors in the animals with similar growth rates during the 3 week period, suggesting that RhoC does not have an effect on tumorigenesis or tumor growth. On the other hand, the clones stably expressing RhoC showed a significantly higher incidence of lung metastases compared with the parental cell line and the vector only clones (Fig. 1*B*). These results strongly suggest that RhoC can promote the metastatic process of prostate cancer cells without affecting tumorigenicity *in vivo*. We also examined the effect of RhoC on the growth of these cells *in vitro*. The results of a colorimetric assay after 72 h indicate that there was no significant difference in the growth rate between the cells with and without RhoC (Supplementary Fig. S1*A*). We then examined the rate of DNA synthesis of the cells with and without the expression of RhoC and found that there was no significant difference between these cells (Supplementary Fig. S1*B*). Furthermore, we established a human prostate cell line, PC3MM/tet/RhoC, which contains the tetracycline inducible *RhoC* gene, as well as PC3 cell lines that did or did not ectopically express RhoC. We then examined the rate of cell growth and DNA synthesis of these cells. Again, we found that RhoC did not affect the rate of proliferation of the cells (Supplementary Fig. S1*A* and *B*), which further supports our notion that RhoC has no apparent role in the growth of prostate cancer cells, although it significantly promotes tumor metastasis.

RhoC expression is significantly increased with the advancement of human prostate cancer. To further corroborate our results in a clinical setting, we examined the status of RhoC expression and its relationship with different clinicopathologic factors in prostate cancer by immunohistochemical analysis of 63 prostate tumor specimens. They were randomly selected from surgical pathology archives dating from 1988 to 2001. As shown in Fig. 2*A* and *B*, the expression of RhoC was found to be strongly elevated in high grade tumors, particularly in specimens from patients with metastatic disease, compared with normal prostatic tissue or low grade tumors. The results of our statistical analyses indicate that RhoC is strongly expressed in tumors with higher Gleason grade, although the correlations are not statistically significant (Fig. 2*B*). Importantly, the RhoC expression showed significant positive correlation with the metastases status of the patients ($P = 0.028$). It was also noted that RhoC expression showed a significant inverse correlation to that of NDRG1 ($P = 0.02$), which



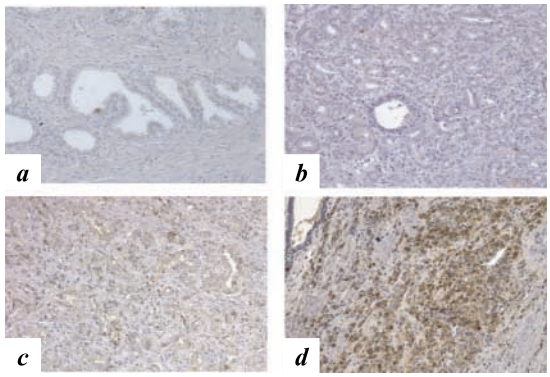
B

Cell line	RhoC*	Tumor incidence†	*In vivo* doubling time	Lung metastasis (mean ± S.E.)‡	*P*
AT2.1	–	7/7	4.1 ± 0.5	3.1 ± 1.3	
Vector only	–	4/4	3.9 ± 0.3	2.0 ± 1.1	
RhoC no. 6	+	8/8	3.9 ± 1.1	10.9 ± 3.5	0.04§
RhoC no. 10	+	5/5	3.8 ± 0.7	9.4 ± 2.4	0.03§

* RhoC expression was examined by Western blot.
† Number of tumor-bearing SCID mice / no. of tumor-inoculated SCID mice.
‡ Number of metastatic lesions on lungs per SCID mouse.
§ $P < 0.05$.

Figure 1. RhoC promotes tumor metastasis without affecting the primary tumor growth *in vivo*. The RhoC expression plasmid was introduced into a low metastasis rat prostate cell line, AT2.1, and clones (no. 6 and no. 10) that constitutively express RhoC were established. As a control, the original vector was also cloned into AT2.1. These clones, as well as the parental line, were injected s.c. into SCID mice as described previously. The volume of the primary tumor for each clone at the indicated time was measured using the equation: volume = (width + length) / 2 × W × L × 0.5236 (*A*). *Inset,* results of a Western blot of RhoC expression for each clone. Mice were sacrificed 3 wk after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically (*B*). §, $P < 0.05$, statistically significant difference.

A



B

Relationship between RhoC and other clinical variables

Factor	All (63)	RhoC expression		*P*
		Positive (33)	Reduced (30)	
Age (y)				
> 71	41	20 (48.78%)	21 (51.22%)	
≤ 70	22	13 (59.09%)	9 (40.91%)	0.605
Gleason grade				
≥ 7	33	21 (63.64%)	12 (36.36%)	
< 7	30	12 (40%)	18 (60%)	0.104
Androgen receptor				
Positive	50	29 (58%)	21 (42%)	
Negative	13	4 (30.77%)	9 (69.23%)	0.150
PTEN				
Positive	39	18 (46.15%)	21 (53.85%)	
Reduced	24	15 (62.5%)	9 (37.5%)	0.316
NDRG1				
Positive	40	16 (40%)	24 (60%)	
Reduced	23	17 (73.91%)	6 (26.09%)	0.02*
Metastasis status				
Organ-confined	34	14 (41.18%)	20 (58.82%)	
Lym/bone	26	19 (73.08%)	7 (26.92%)	0.028*

Figure 2. Immunohistochemical analysis of RhoC in human prostate cancer. Immunohistochemical staining was performed on paraffin embedded human prostate tissue sections using anti RhoC antibody and the results were compared with other clinical variables. *A,* representative field with immunostaining for RhoC in normal prostate tissue (*a*), low grade carcinoma (*b*), high grade localized carcinoma (*c*), and high grade metastatic carcinoma tissue (*d*). *B,* association of RhoC with other clinical variables was analyzed by standard χ^2 test using SPSS software. *, $P < 0.05$, statistically significant difference.

has recently been shown to be a tumor metastases suppressor in prostate cancer (19). These results suggest that the expression of RhoC is up regulated at a relatively late stage and is directly involved in metastatic progression of prostate cancer, which is in good agreement with our *in vivo* data. Furthermore, the results of our survival analyses on 50 patients with prostate cancer over a period of 5 years indicates that patients with positive expression of RhoC had significantly worse overall survival rate than the patients with a reduced expression of the gene ($P = 0.018$, log rank test; Fig. 3). The results of univariate Cox regression analysis revealed that the death risk of patients with increased RhoC expression was 4.8 times higher than the risk of patients with RhoC negativity. However, when we performed a multivariate analysis for RhoC, Gleason score, and metastasis, only the metastasis status gave a significant value ($P = 0.015$) and other two factors were excluded. The fact that multivariate analyses of these three factors excluded RhoC status indicates that the profiles of the RhoC expression and metastasis status of patients significantly overlaps and that each factor has enough "power" for predicting patient outcome. In fact, when we did a multivariate analysis for a combination of RhoC status and Gleason score, which is the most widely used pathologic marker for prostate cancer, RhoC status turned out to be a better predicting marker than Gleason score ($P = 0.037$ and $P = 0.237$ for RhoC and Gleason score status, respectively). Although RhoC expression did not significantly and independently predict survival compared with metastasis, increased RhoC correlates with aggressive disease which could account for increased metastatic disease.

RhoC promotes invasiveness and motility of prostate cancer cells *in vitro*. To understand how RhoC contributes to the progression of prostate cancer, we ectopically expressed the *RhoC* gene in the human prostate cancer cell line, PC3, followed by examining the invasiveness and migration of the cells *in vitro*. We found that the expression of RhoC significantly enhanced both cell

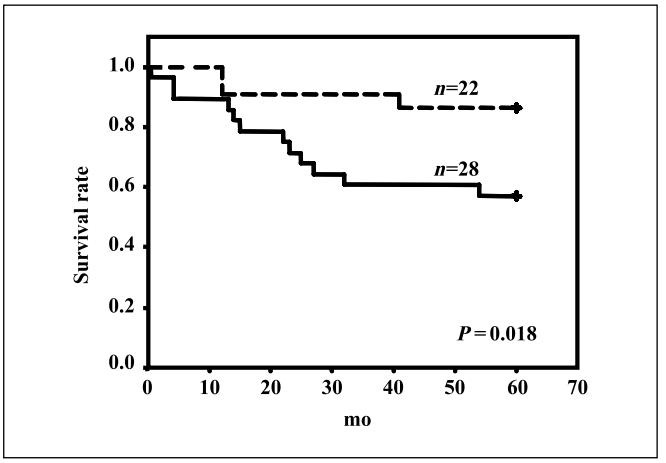


Figure 3. Prognostic value of RhoC expression. Overall survival rate over a period of 5 y was calculated in 50 patients with prostate cancer in relation to the expression of the *RhoC* genes by Kaplan Meier method. $P = 0.018$ was determined by a log rank test. RhoC positive (*solid line*) patients and patients with reduced expression (*dotted line*) of RhoC.

invasiveness and migration ($P = 0.03$ and 0.004, respectively; Fig. 4*A*), which is in good agreement with the previous results of Yao and colleagues (20). The effect of RhoC on cell motility was also examined by the "wound healing" assay. As shown in Fig. 4*B*, cells with ectopically expressing RhoC showed a much higher rate of motility compared with the cells with an empty vector transfectant. These results strongly suggest that RhoC promotes metastasis by enhancing the invasiveness and/or motility of tumor cells. Because the invasive ability of tumor cells is known to often be correlated with their production of secretory proteases (21), we examined the expression of MMP2 and MMP9 in the cells that overexpressed RhoC. As shown in Fig. 4*C*, quantitative reverse transcription PCR (qRT PCR) analysis for the cell overexpressing RhoC significantly augmented the level of the expression of the *MMP2* and *MMP9* genes ($P = 0.049$ and 0.02, respectively). These results were further validated by gelatin zymography and Western blot analyses as shown in Fig. 4*D*. Therefore, our results indicate that the invasiveness of tumor cells induced by RhoC is, at least in part, due to the overexpression of MMP2 and MMP9.

RhoC activates MMP through the Pyk2 signal pathway. To gain further insight into the signaling pathways by which RhoC promotes the invasive phenotype, we prepared cell lysates from PC3MM/tet/RhoC with or without induction of the *RhoC* gene by tetracycline. The lysates were labeled with Cy3 and Cy5 and analyzed on an antibody microarray which contained 224 anti bodies for various key molecules of cell signaling and cell cycle, and the results of ratios were rank ordered. As shown in Fig. 5*A* (*left*), ectopic expression of RhoC significantly phosphorylated a series of protein kinases including MAPK, FAK, Akt, and Pyk2. The result of the array analysis was also confirmed by Western blot using the antibodies specific to phosphorylated proteins as well as the antibodies to the total proteins for each signal molecule (Fig. 5*A, right*; Supplementary Fig. S2*A*). These results suggest that RhoC can directly activate a cascade of signal pathways involving these key signal molecules that are closely related to cell motility and tumor progression.

Pyk2 is a tyrosine kinase and belongs to a member of the FAK subfamily which plays a critical role in cell migration and motility of various cell types (22, 23). Pyk2 is also known to be able to phosphorylate Akt (23). Therefore, we investigated the possibility

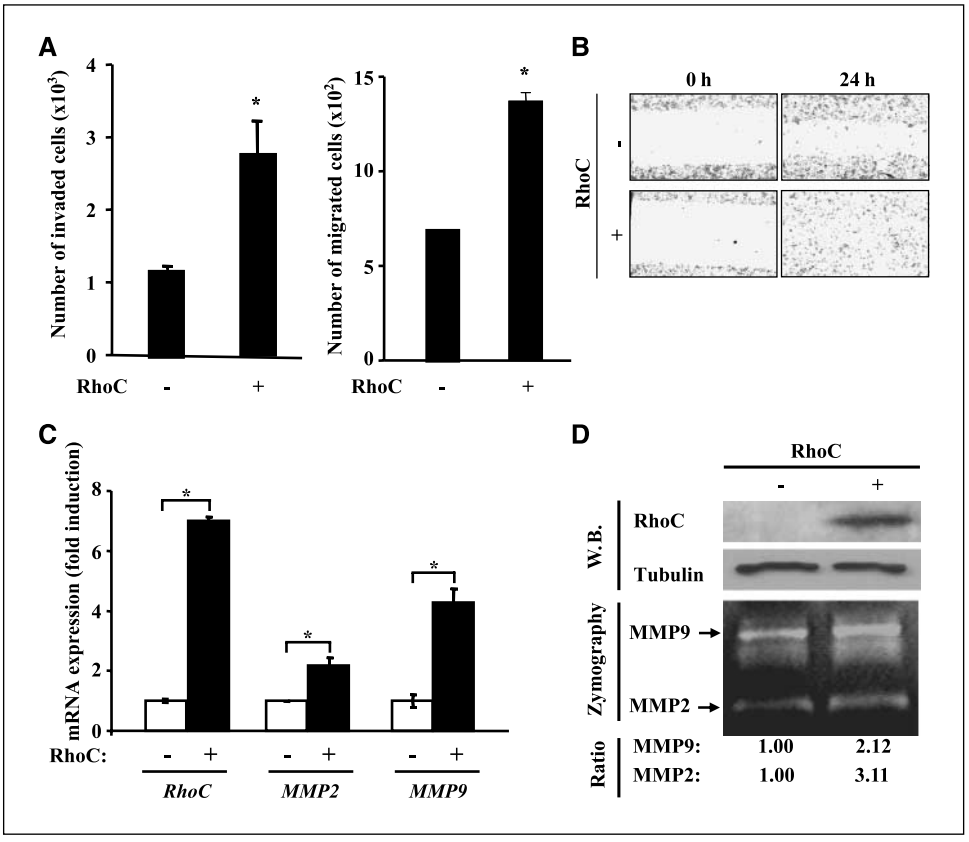
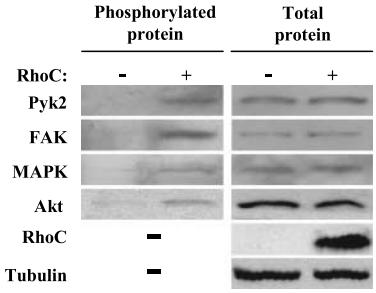


Figure 4. RhoC promotes invasiveness and motility of prostate cancer cells *in vitro. A,* the RhoC expression plasmid (pcDNA3/RhoC) or the vector alone was transfected into the PC3 cell line. After 24 h, cells were collected and subjected to invasion (*left*) and migration (*right*) assays. *, $P < 0.05$, statistically significant difference. *B,* for the motility assay, the PC3 cells stably transfected with the RhoC expression plasmid or an empty vector were cultured to confluency. The monolayer was scratched by drawing lines and photographed under a microscope. After 24 h of incubation, they were photographed again. *C,* to test the effect of RhoC on MMP2 and MMP9, PC3 cells that have been stably transfected with the RhoC expression plasmid or an empty vector were cultured in 12 well plates. Cells were then collected and their total RNA was treated with DNase. The RNA was then subjected to qRT PCR using specific primers for the *RhoC, MMP2*, and *MMP9* genes. Results were presented as ratios of the expression level of each gene in RhoC positive and RhoC negative cells. *, $P < 0.05$, statistically significant difference. *D,* MMP2 and MMP9 activities in the conditioned medium from the PC3 cells with or without the RhoC expression plasmid as described in *C* were assayed by gelatin zymography. The image was photographed and the intensity of each band was digitally quantified. The expression of Flag RhoC was confirmed by Western blot (*top*).

A

Gene Name	Phosphorylation site	Ratio (Cy5/Cy3)
MAPK	Thr^{183}	2.85
FAK	Ser^{910}	2.47
PKB/Akt	Thr^{308}	2.41
FAK	Tyr^{577}	2.38
FAK	Ser^{722}	2.35
MAPK	Thr^{183}/Tyr^{185}	2.35
FAK	Ser^{473}	2.31
PyK2	Tyr^{881}	2.26
MAPK	Tyr^{185}	2.21
PAK	Ser^{212}	2.2
PyK2	$Tyr^{579/580}$	2.2
FAK	Tyr^{397}	2.14



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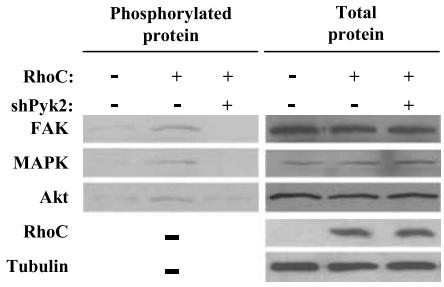

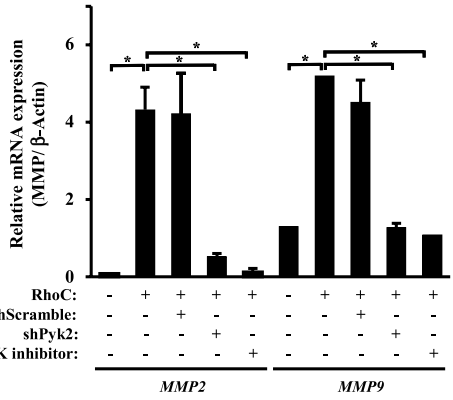

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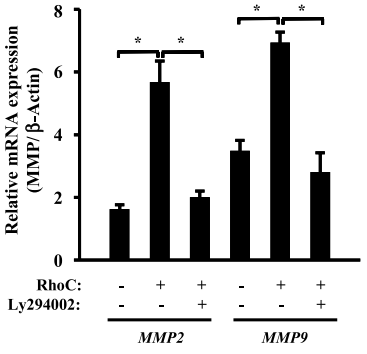

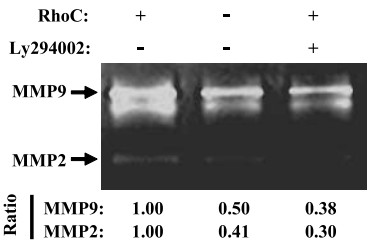

D

	RhoC expression		
All (27)	Positive (13)	Reduced (14)	*P*
P-Akt (Ser^{473})			
Positive (14)	10	4	
Reduced (13)	3	10	0.033*

E

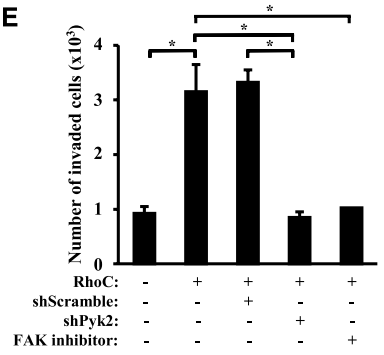

Figure 5. RhoC activates MMPs through the Pyk2/FAK pathway. *A,* for antibody array analysis, cell lysates were prepared from the PC3MM/tet cells containing the tetracycline inducible *RhoC* gene with or without induction of RhoC. The proteins were labeled with Cy3 or Cy5 and subjected to antibody microarray (Sigma Aldrich) analysis. The scanned data was analyzed by GenePix Pro 5.0 (Axon Instrument). The result of the antibody array data was confirmed by Western blot using phosphospecific antibodies to Pyk2, FAK, MAPK, and Akt as well as using antibodies to the total protein of each corresponding gene. *B,* PC3 cells stably transfected with the RhoC expression plasmid or an empty vector were transfected with the expression plasmid of shRNA for *Pyk2* or a scrambled sequence. After 48 h, cells were collected and subjected to Western blot analysis using phospho specific antibodies (*left*). To examine the effect of Pyk2 and MAPK on the MMP expression, the same set of cells were treated with or without the MAPK inhibitor, PD98059 (100 μmol/L) for 48 h. RNA was extracted from each sample (in triplicate) and subjected to qRT PCR using specific primers for *MMP2* and *MMP9* (*right*). *C,* the effect of Akt phosphorylation on MMP expression was examined. Cells with or without expression of RhoC were treated with or without PI3K/Akt inhibitor, Ly294002 (100 nmol/L), for 48 h. The cells were then collected and RNA was extracted followed by qRT PCR analysis for *MMP2* and *MMP9* expression (*left*). The conditioned culture mediums of the same set of samples were subjected to zymography assay for MMP2 and MMP9 (*right*). The image was photographed and the intensity of each band was digitally quantified. *D,* to examine the clinical status of RhoC and p Akt expression, 27 samples from patients with prostate cancer were analyzed by immunohistochemistry using antibodies to RhoC and p Akt. The result was analyzed by χ^2 test. *E,* PC3 cells with or without RhoC expression were treated with shPyk2 or the FAK specific inhibitor, TAE226, for 48 h. The cells were then assayed for their invasiveness by using a Matrigel invasion assay as described in Materials and Methods.

that Pyk2 is an immediate effector of the RhoC signal and that it controls the downstream pathways. PC3/RhoC cells were transfected with the expression vector of shRNA targeted to *Pyk2*. After 48 h of incubation, cell lysates were prepared and subjected to Western blot analysis using antibodies to RhoC, p FAK, p MAPK, and p Akt. As shown in Fig. 5*B* (*left*) and Supplementary Fig. S2*B*, induction of RhoC strongly phosphorylated FAK, MAPK, and Akt, and this RhoC dependent phosphorylation of these molecules was strongly blocked by the addition of shRNA to the *Pyk2* gene, suggesting that RhoC first activates Pyk2, which then phosphorylates FAK, MAPK, and Akt. We then examined whether MMP2 and MMP9 are indeed activated by Pyk2 and MAPK in a RhoC dependent manner. RNA was prepared from PC3/RhoC cells that were cultured in the presence or absence of shRNA for *Pyk2* and the MAPK inhibitor, PD98059. RNAs were then examined for the expression of *MMP2* and *MMP9* by qRT PCR. As shown in Fig. 5*B* (*right*) and Supplementary Fig. S2*C* (*left*), RhoC dependent activation of both MMP2 and MMP9 was significantly abrogated in the presence of shRNA for *Pyk2* or the MAPK inhibitor, suggesting that the activation of MMP2 and MMP9 by RhoC is at least partly due to the phosphorylation of Pyk2 followed by the activation of MAPK. Because our results indicate that Akt is also phosphorylated at Ser^{473} by RhoC in a Pyk2 dependent fashion, we examined whether Akt is also involved in the activation of MMP2 and MMP9 in the RhoC signal pathway. As shown in Fig. 5*C* (*left*) and Supplementary Fig. S2*C* (*right*), we found that the RhoC dependent induction of *MMP2* and *MMP9* was indeed significantly blocked by PI3K/Akt inhibitor, Ly294002. This result was further confirmed by gelatin zymography analysis as shown in Fig. 5*C* (*right*). To further corroborate the *in vitro* results, we examined 27 clinical specimens from patients with prostate cancer by conducting immunohistochemistry using anti RhoC and anti phospho Akt (Ser^{473}) antibodies. As shown in Fig. 5*D*, we found that RhoC expression was significantly correlated with the expression of phospho Akt in these tumor tissues. Therefore, these clinical data as well as the *in vitro* results strongly suggest that Akt is part of the downstream effectors of RhoC signals and plays an important role in RhoC dependent activation of MMP2 and MMP9. To further validate the role of Pyk2 and FAK in the RhoC induced signal, we treated the PC3 cells that do or do not express RhoC with shPyk2 or the FAK specific inhibitor, TAE226, followed by measuring the invasiveness of these cells using the Matrigel invasion chamber assay. As shown in Fig. 5*E*, we found that inhibition of Pky2 and FAK indeed significantly blocked the RhoC induced invasiveness of the prostate tumor cells, which strongly suggests the functional involvement of Pyk2 and FAK in the RhoC signaling pathway.

Discussion

RhoC has been shown to be involved in various types of tumors (9 11). However, the exact role of RhoC in tumor progression and its underlying mechanism are unclear, and the previous results from different groups have presented an apparently contradictory picture of the function of this gene (12 15). In this study, we have integrated multiple approaches, both *in vitro* and *in vivo*, to clarify the functional role of RhoC in prostate cancer progression. The results of our animal experiments clearly indicate that RhoC plays a critical role in the metastatic progression of prostate tumor but it is not essential for tumor cell growth. The results of immunohistochemical analysis of human prostate cancer specimens also indicates that RhoC expression is significantly correlated with the metastatic status of the patients but not with Gleason grade, which strongly supports our notion that RhoC is implicated mainly in the metastatic process but not in tumorigenesis. Importantly, RhoC expression is inversely correlated with patient survival, suggesting that RhoC can serve as a prognostic marker as well as a potential therapeutic target for prostate cancer.

The molecular mechanism by which RhoC promotes tumor progression is an intriguing question. We have constructed a RhoC inducible cell line and examined its protein expression profile using an antibody array to clarify the signal pathway. The results of the array analysis revealed that Pyk2, FAK, MAPK, and Akt were all phosphorylated upon induction of the RhoC expression, and the knockdown of *Pyk2* resulted in significant reduction in phosphorylation of FAK, MAPK, and Akt, suggesting that Pyk2 is the upstream effector and plays a central role in the RhoC signal pathway. Pyk2 belongs to the subfamily of focal adhesion protein tyrosine kinases and it has been shown to be involved in cell migration, invasion, and proliferation (24 28). It was reported that in the *in vitro* model of transforming growth factor β induced epithelial to mesenchymal transition, Pyk2 was strongly phosphorylated at Tyr^{881} whereas during migration, Pyk2 was strongly phosphorylated at Tyr^{580} (22). It should be noted that, in our antibody array analyses, both of these sites were found to be phosphorylated (Fig. 5*A*). Pyk2 is capable of transducing signals via several known pathways, and one of the effectors is FAK which has been shown to be phosphorylated by Pyk2 at Tyr^{397}, $Tyr^{576/577}$, and Tyr^{925} (29). The results of our antibody array data also revealed that both of these sites were indeed phosphorylated upon induction of RhoC. These results suggest that RhoC activates FAK via phosphorylation of Pyk2. FAK is a focal adhesion kinase and plays a critical role in cell migration and motility (30 32). The enhanced expression of FAK has been documented in a number of different types of human cancers (33 41). The phosphorylation of FAK is known to be linked to the activation of several downstream signals including ERK and JNK/MAPK as well as PI3K/Akt (42, 43). Furthermore, it was previously shown that the invasive ability of RhoC was significantly attenuated by a MAPK inhibitor *in vitro* (44). Notably, the results of our knockdown experiments using *Pyk2* specific shRNA has shown that the RhoC dependent phosphorylation of both ERK/MAPK and Akt was significantly blocked by knockdown of *Pyk2*, suggesting that MAPK and Akt are activated by RhoC via phosphorylation of Pyk2 and FAK.

We have shown that RhoC promotes metastasis by augmenting the motility and invasion of tumor cells (Figs. 4 and 5) via activation of MMP2 and MMP9, two key proteases for the invasion of tumor cells. It should be noted that the expression of both MMP2 and MMP9 was previously shown to be modulated by the activation of Akt and MAPK (45 47). We have indeed shown that inhibitors of both molecules significantly blocked the RhoC dependent activation of MMP2 and MMP9. In this context, it should be noted that Ruth and colleagues have recently shown that RhoC promoted the invasion of human melanoma cells in a PI3K/Akt dependent manner (48). Our results also indicate that Akt was significantly phosphorylated at Ser^{473} by RhoC, and that the phosphorylation of this serine residue has previously been found to be involved in the motility and invasiveness of tumor cells (45, 46, 49). The activation of Akt has also been shown to be clinically associated with aggressiveness and earlier recurrence of prostate cancer (50). Collectively, our results indicate that RhoC enhances the invasiveness and metastatic ability of tumor cells by

activating the Pyk2/FAK pathway followed by phosphorylation of Akt and MAPK, which in turn, activate MMP2 and MMP9. RhoC is considered to serve as an independent prognostic marker to predict patient outcome, and an intervention of the RhoC signal may be an effective therapeutic strategy for prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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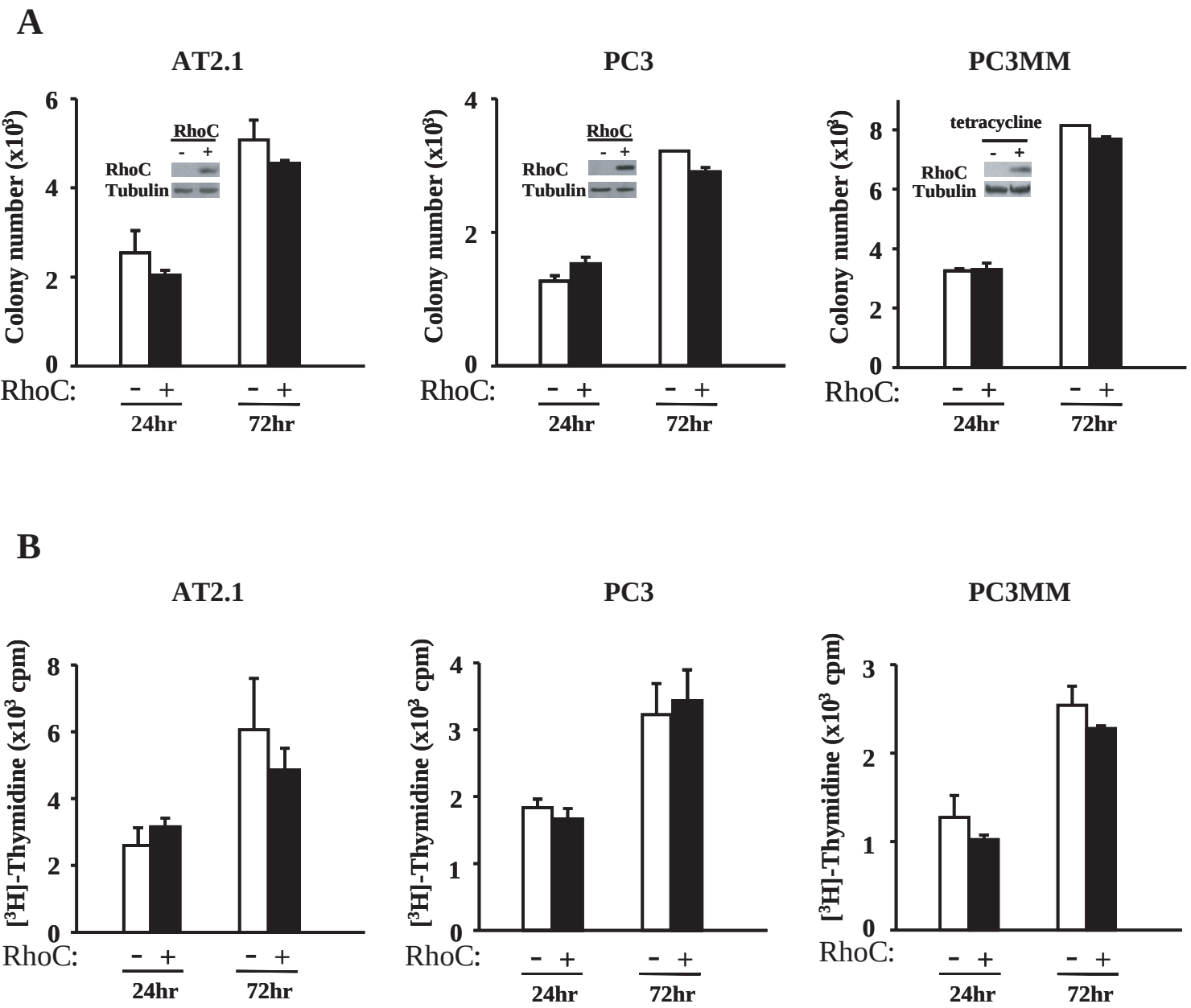
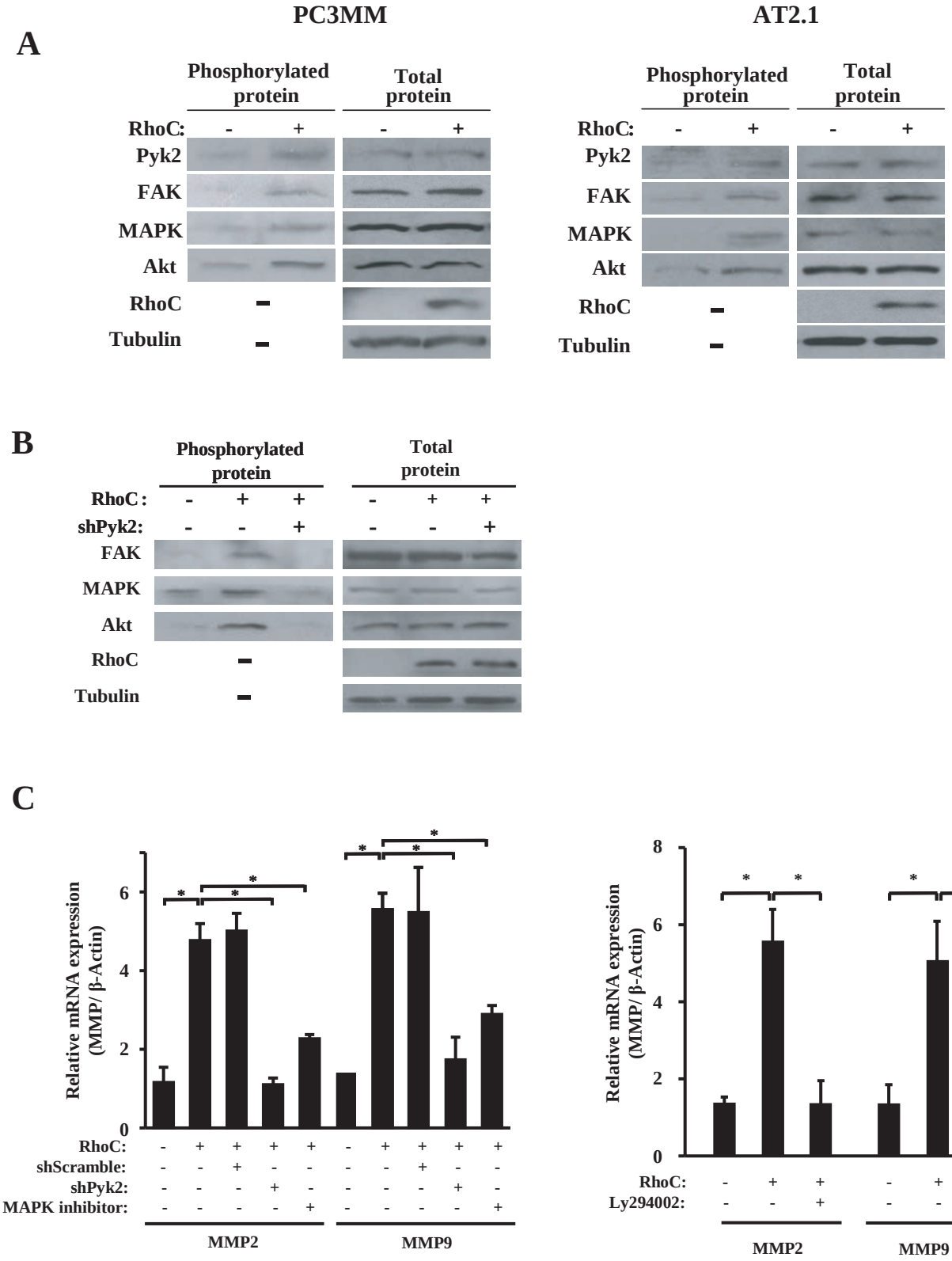


Supplementary Fig. S1 Iiizumi et al



Supplementary Fig. S2 Iiizumi et al